TYPE I PROCOLLAGEN PROCESSING IN THE DEVELOPING CHICK

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Tsar Dadon began to dream
A quiet ending to his labours

A. S. Pushkin, *The Tale Of The Golden Cockerel*

CONTENTS

ABSTRACT OF THESIS

It is not known what factors regulate collagen fibril diameter *in vivo*. Proposed factors include the presence of proteoglycans or glycosaminoglycans, the amount of post-translational glycosylation of hydroxylysyl residues in the collagen molecule and copolymerization of other collagen types in the same fibril. Another proposed factor is the route of extracellular removal of the N- and C-terminal propeptides from the biosynthetic precursor of collagen, procollagen, by the procollagen N- and C-proteinases. Processing via a pN-collagen intermediate has been proposed to result in narrow fibrils and processing via a pC-collagen intermediate was proposed to lead to wide fibrils. A pulse-chase system was devised in order to measure the rate constants of the reactions of procollagen processing in chick tendon and cornea at different stages of embryonic development. Pulse-chase data were analyzed by SDS-PAGE, fluorography and densitometry, and the densitometric data were used in a computer program to determine the rate constants of processing. The results showed that the initial cleavage at either end of the procollagen molecule in both tendon and cornea was random. The values of the rate constants of processing differed in cornea and tendon, and at each stage of embryonic development studied. The results were inconsistent with a direct role of procollagen processing in the control of collagen fibril diameter, and suggested that regulation of the processing enzymes in the two tissues is different, or that different enzymes may be responsible for cleaving the two procollagens.

An *in vitro* study exposing purified type I procollagen from tendon or cornea to the purified N- and C-proteinases was used to simulate the process of collagen fibril formation. It was found that although both procollagen samples were completely processed by the enzymes, the corneal collagen did not aggregate in the form of fibrils

whereas the tendon collagen did. It was proposed that this was due to increased solubility of the corneal collagen. Initial attempts to investigate the levels of post-translational glycosylation of the tendon and corneal procollagens by lectin blotting are described, to see if the increased solubility was due to the presence of greater amounts of hydroxylysyl glycosides in corneal collagen.

Declaration

This thesis has been composed solely by myself, and all the work described herein is my own, except where stated in the text.

Sally J Mellor

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ABBREVIATIONS

Amino Acids

Amino acids are generally referred to by their 3-letter code:

Ala	Alanine
Asn	Asparagine
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Other amino acids:

Hypro	Hydroxyproline
Hylys	Hydroxylysine

Other Abbreviations

α-chain	Individual polypeptide chain of tropocollagen
AMPS	Ammonium persulphate
ATP	Adenosine triphosphate
BAPN	β-Aminopropionitrile
Bq	Becquerel(s)
BSA	Bovine serum albumin
C-propeptide	Carboxy-terminal globular domain of procollagen
C-terminal	Carboxy-terminal
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CD	Circular dichroism
cDNA	Complementary DNA
Ci	Curies
CNBr	Cyanogen bromide
Col 1	Amino-terminal fragment of dermatosparactic sheep proα1(I) chain obtained after collagenase treatment
COL 1-3	Triple helical domains of type IX collagen
cpm	Counts per minute
CS	Chondroitin sulphate
CS-GAG	Chondroitin sulphate glycosaminoglycan
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DON	6-Diazo-5-oxo-L-norleucine
DS	Dermatan sulphate
DS-GAG	Dermatan sulphate glycosaminoglycan
DS-PG	Dermatan sulphate proteoglycan
ΔG	Gibbs free energy change
ΔH	Enthalpy change

ΔS	Entropy change
EC1-3	Chains of type VIII collagen
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EDTA	Ethylenediamine tetraacetic acid, disodium salt
EM	Electron microscopy
ER	Endoplasmic reticulum
f	Frictional coefficient
f_{min}	Minimum frictional coefficient for unsolvated rigid spheres
FCS	Foetal calf serum
Fe^{2+}	Ferrous ions
GAG	Glycosaminoglycan
glc	Gas-liquid chromatography
^3H	Tritium
HA	Hyaluronic acid
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HS	Heparan sulphate
k_{cat}	Turnover number of an enzyme
kBq	Kilobecquerel(s)
KCl	Potassium chloride
K_D	Dissociation constant
KH_2PO_4	Potassium dihydrogen phosphate
K_i	Inhibition constant
K_m	Michaelis constant
KS	Keratan sulphate
KSPG	Keratan sulphate proteoglycan
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
MBq	Megabecquerel(s)
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
min SSQ	Minimum of the sums of squares of the residuals
ml	millilitre(s)
mmol	millimole(s)
mRNA	Messenger RNA
μg	Microgram(s)
μM	Micromolar
μm	Micrometre(s)
N-propeptide	Amino-terminal propeptide of type I procollagen
N-terminal	Amino-terminal
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
$NaHPO_4$	Sodium hydrogen phosphate
NaH_2PO_4	Disodium hydrogen phosphate
NaN_3	Sodium azide
NC1-4	Non triple helical domains of type IX collagen
NEM	N-ethyl maleimide
NHS-biotin	N-hydroxysuccinimidobiotin
nm	Nanometre(s)
O-linked	Ester linkage
OI	Osteogenesis imperfecta

PBS	Phosphate buffered saline
pC	pC-collagen: procollagen with the N-propeptide absent
PDI	Protein disulphide isomerase
PEG	Polyethylene glycol
PG	Proteoglycan
PMSF	Phenylmethylsulphonylfluoride
pN	pN-collagen; procollagen without the C-propeptide
PPO	2,5-Diphenyloxazole
Proα1	α1 chain of procollagen
Proα2	α2 chain of procollagen
PS	Penicillin-streptomycin
PTA	Phosphotungstic acid
rpm	Revolutions per minute
^{35}S	Sulphur-35
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SLS	Segment-long-spacing
SRP	Signal recognition particle
TBq	Terabecquerel(s)
TC_a	Large N-terminal fragment of collagen produced by collagenase cleavage
TC_b	C-terminal fragment produced by collagenase cleavage
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFβ	Transforming Growth factor-β
TIMP	Tissue Inhibitors of Metalloproteinases
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
UA	Uranyl acetate
V_{max}	Maximal velocity of an enzyme
Zn^{2+}	Zinc ions

CHAPTER 1

INTRODUCTION

1.1 The Extracellular Matrix

The cells of connective tissues such as skin, tendon, cartilage or blood vessel walls produce a matrix in which they are embedded (Hay, 1981). This acellular material is known as the extracellular matrix (ECM), and it consists of molecules such as collagen, elastin, proteoglycans (PG), glycosaminoglycans (GAG) and glycoproteins such as fibronectin and laminin (for reviews, see Heathcote and Grant, 1980; Hardingham, 1981; Hay, 1981; Sandberg, Soskel and Leslie, 1981; Muir, 1983; Hukins and Aspden, 1985; Poole, 1986; Paulsson, 1987; Ruoslahti, 1988).

1.2 The Collagens

The collagens are the major protein components of the extracellular matrix, and constitute the most abundant protein in the human body (Martin et al, 1985). There are at least twelve, and possibly thirteen, genetic types of collagen characterized so far (Mayne and Burgeson, 1987). The molecules of the most abundant collagens, I, II and III, assemble to form fibrils. These fibrils are long (>10 μm), approximately circular in cross-section and periodically banded. Other collagens form a variety of supramolecular structures. Collagens contribute to a variety of functions, e.g. tensile strength in tendons, optical transparency in cornea, provision of a framework for calcification in bone and filtration in the glomerular basement membrane. The molecular structure of collagens and their various modes of packing allows them to carry out these functions.

Twelve genetic types of collagen have been characterized, and these differ genetically, chemically, and immunologically (Cheah, 1985; Martin et al, 1985; Dublet and van der Rest, 1987; Gordon, Gerecke and Olsen, 1987; Mayne and Burgeson, 1987). A convenient

Type	Chains	Triple Helix Length	Aggregated Form	Localization
Fibrillar Collagens				
I	α1(I), α2(I)	300 nm	67-nm periodic fibrils	Skin, tendon, bone, cornea
II	α1(II)	300 nm	67-nm periodic fibrils	Cartilage, vitreous humour
III	α1(III)	300 nm	67-nm periodic fibrils	Skin, muscle
V	α1(V), α2(V) α1'(V), α3(V)	310 nm	Small fibres	Most interstitial tissues
XI	α1(XI), α2(XI) α3(XI)	310 nm	Small fibres	Cartilage
Non-fibrillar Collagens, length > 300 nm				
IV	α1(IV), α2(IV)	390 nm	Non-fibrillar network	All basement membranes
VII	α1(VII)?	450 nm	Dimeric aggregates	Anchoring fibrils
Non-fibrillar Collagens, length < 300 nm				
VI	α1(VI), α2(VI), α3(VI)	150 nm	Microfibrils, 100-nm banded fibrils	Most interstitial tissues
VIII	α1(VIII)	?	?	Some endothelial cells, Descemet's membrane
IX	α1(IX), α2(IX), α3(IX)	200 nm	?	Cartilage, vitreous humour
X	α1(X)	150 nm	?	Hypertrophic and mineralizing cartilage

Table 1.1 Classification of collagen types according to those which form fibrils and those which do not form fibrils. Type XII is omitted from this table, as its position in this classification is not known. After Miller, 1985.

classification for the purposes of this thesis is a distinction between the types of collagen which form fibrils, and those which do not form fibrils, of which there are two categories (Miller, 1985; Table 1.1). A diagrammatic representation of molecular structure is shown in Figure 1.1. Of the fibrillar collagens, types I,II and III consist of a long central triple-helical domain 300 nm long and 1.5 nm wide of molecular weight 285,000 daltons. Types II and III differ from type I in their amino acid composition and extent of post-translational modifications. Type V and type XI collagen are equivalent to types I,II, and III, in so far as they also appear to contain a long triple helical domain 300 nm long.

Type V collagen is present in many tissues apparently as non-striated fibrils, but it can also coexist with type IV collagen in basement membranes (for a review, see Fessler and Fessler, 1987). There appear to be four types of α(V) chain: α1(V), α1'(V), α2(V) and α3(V), which seem to differ in propeptide size (Fessler and Fessler, 1987). The role of type V collagen may be to limit the size of type I collagen fibrils (Adachi and Hayashi, 1985, 1986; see Section 1.6.4.3; Birk *et al*, 1988).

Type XI collagen was first discovered during differential fractionation of pepsin digests of hyaline cartilage, and migrates on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as three distinct α chains which migrate more slowly than α(II) chains. Type XI appears to exist as a heterotrimer of three α chains, α1(XI), α2(XI) and α3(XI) (for a review of type XI collagen, see Eyre and Wu, 1987). Type XI appears to be present as a minor constituent in all tissues in which type II is present as the main type (Eyre and Wu, 1987). The actual polymeric form of type XI collagen is still uncertain, but it could possibly form heteropolymeric fibrils with type II collagen. It can form non-striated fibrils *in vitro* (Brodsky and Eikenberry,

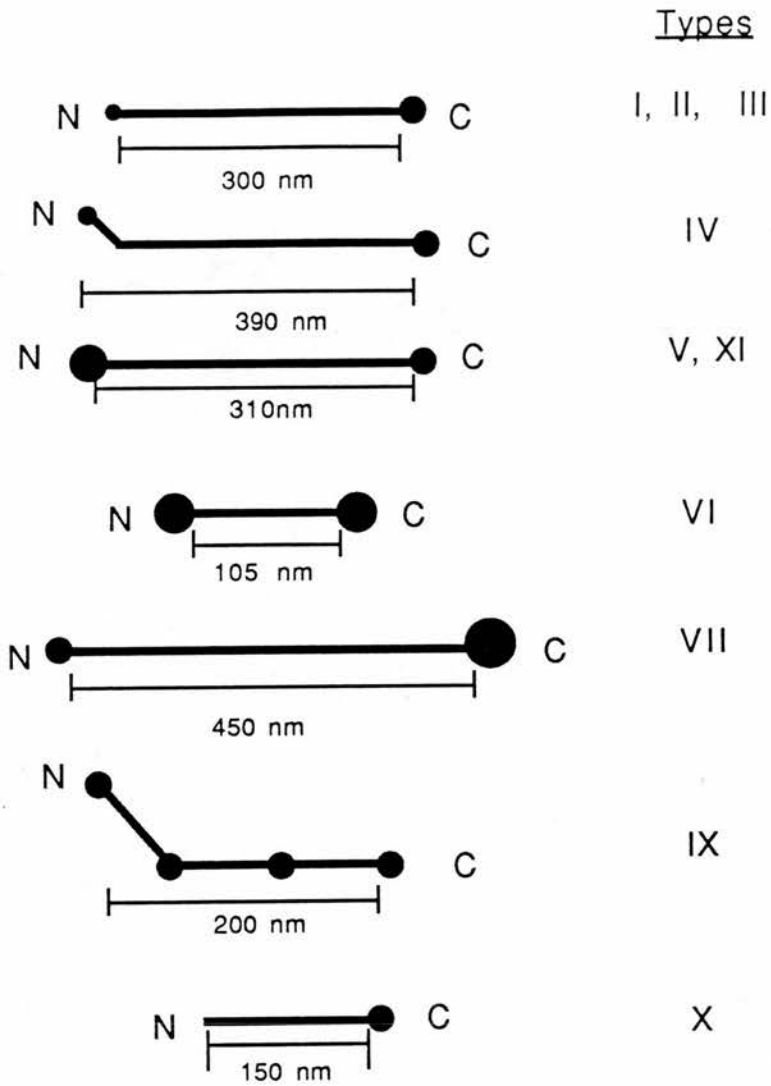

Figure 1.1 Schematic representation of the molecular structures of the various collagen types. The triple helical domains are represented by rods, non helical domains by closed circles. The lengths of the triple helical regions are given in nm, but the lengths are not drawn to scale. The relative sizes of the propeptides are indicated, i.e. C-propeptide larger than the N-propeptide and so on. The N-terminal and C-terminal ends of the molecule are indicated.

The molecular structure of type VIII collagen is unknown. The N-propeptide of proα2(V) is either very small, or absent.

1985). The apparently close similarities of types V and XI collagen suggests that type XI collagen might have a role in type II fibrillogenesis analogous to that proposed for type V in type I fibril formation (Section 1.6.4.3). Another possible function proposed for type XI collagen could be in mediating collagen-PG interactions. Type XI in the form of native molecules in solution or of reconstituted fibrils binds to cartilage PGs, whereas type II does not bind. If this binding occurred in vivo then type XI could be functioning as an anchor to attach cartilage collagen fibrils to cartilage matrix PGs or to sulphated GAGs in the cell membrane (Eyre and Wu, 1987).

The second category of collagen types proposed by Miller (1985) is where the collagens exist in a non-fibrillar form with a triple helical length of greater than 300 nm, and types IV and VII fall into this category (Table 1.1).

Type IV collagen exists only in basement membranes (Glanville, 1987). Rotary shadowing shows a triple helical domain 360 nm in length with a short (30 nm) arm at the amino (N)-terminus, and a globular carboxy (C)-terminus. Two models of aggregation of type IV collagen have been proposed, based on the association of type IV monomers via both N- and C-terminal domains (for a review, see Glanville, 1987). Further association of these aggregates could lead to formation of a "network" structure, with the two different models resulting in networks with different pore sizes. There are two α(IV) chains, α1(IV) and α2(IV), but it is not clear whether the type IV molecule exists as homotrimers or heterotrimers.

The triple helical domain of type VII collagen is about 350 nm in length as shown by rotary shadowing (for review, see Burgeson, 1987). Type VII collagen can form antiparallel dimers stabilized by intermolecular disulphide bonds, which can then condense laterally into an unstaggered array. The overlap of the two molecules in the dimer is thought to be 60 nm. Intact Type VII reveals two globular domains when rotary shadowed. The rotary

shadowing pictures suggest that the large C-terminal domain structure consists of 3 arms. Small N-terminal domains are also present. Type VII collagen appears to be the main component of anchoring fibrils, which secure the lamina densa (underlying certain epithelia) to the subjacent stroma, as a comparison of the banding pattern of anchoring fibrils with those observed for type VII segment-long-spacing (SLS) crystallites (see Section 1.6.1) suggests that the anchoring fibril is composed mainly of type VII collagen. The anchoring fibrils extend perpendicularly from the basement membrane, inserting into "anchoring plaques" within the matrix beneath. Anchoring plaques can be connected to one another by additional anchoring fibrils. These anchoring plaques seem to contain the C-terminal domains of type VII collagen and type IV collagen, forming an extensive "scaffold" throughout the sub-basal lamina (for references, see Burgeson, 1987).

The third class of collagen types proposed by Miller (1985) contains those molecules which are non-fibrillar with triple helical domains of less than 300 nm in length (Table 1.1). This class includes collagen types VI, VIII, IX, X and XII. Intact type VI collagen monomers appear in electron micrographs after rotary shadowing as "dumb-bell" shaped molecules with 2 globular domains of apparently equal size connected by a 105 nm long triple helix. Type VI collagen molecules can assemble into dimers and tetramers which are present in preparations of pepsin-solubilized collagen from human placenta when visualized by rotary shadowing and negative staining. Dimers are formed by a staggered antiparallel arrangement with a 75 nm overlap of the triple helical domains, which gives rise to 30 nm long single triple helical segments at each end of the dimer. Tetramers form by the lateral association of dimers with their ends in register, with crossing over of the outer triple helical segments into a scissors-like structure at one or both ends. These tetramers are stabilized by disulphide bonds. The tetramers and dimers

appear to be able to associate into oligomers, and these oligomers appear to aggregate into microfibrils, although it is not known how this occurs (for a review of type VI, see Timpl and Engel, 1987). In most tissues type VI represents less than 3% of the total collagenous protein, but in human cornea as much as 25% of the collagens may be type VI (Zimmermann et al, 1986). Immunostaining patterns obtained with anti-type VI antibodies locate most of the type VI in the extracellular space, separately from type I and III structures (e.g. Alper and Amenta, 1986).

Type VIII collagen is a major constituent of Descemet's membrane (Labermeier and Kenney, 1983; Bornstein and Sage, 1987). Radiolabelling shows that type VIII is secreted as three chains, EC1 (177,000 daltons), EC2 (125,000 daltons), and EC3 (100,000 daltons). EC2 is the first form to be detected in pulse-chase experiments, with EC1 and EC3 apparent at later chase times. However it is still not known whether EC1, EC2 and EC3 are distinct α chains. The structure of type VIII is not known, but current models based on protease digestion studies indicate that interruptions of < 10,000 daltons exist within the triple helix, and there are discrete collagenous domains of 50,000-60,000 daltons (for a fuller discussion of type VIII, see Bornstein and Sage, 1987).

The domain structure of type IX collagen shows that there are three collagenous (COL1-3) domains and four non-collagenous (NC1-4) domains (Figure 1.1). The molecule consists of three genetically distinct chains, α1(IX), α2(IX) and α3(IX) and these are present in equimolar amounts in a single molecule, based on biochemical analyses of the pepsin resistant fragments. Type IX collagen is a PG, as ^{35}S can be incorporated into the 115,000 dalton chain and can be removed by chondroitinase ABC. The GAG chain is linked to the α2(IX) chain and is attached at the NC3 domain (for a review, see van der Rest and Mayne, 1987). Type IX collagen is found in cartilage matrix with type II collagen and a recent report has shown

type IX collagen can be associated with type II fibrils (Vaughan et al, 1988).

Type X collagen is synthesized by hypertrophic cartilage during endochondral ossification (e.g. Ayad, Kwan and Grant, 1987). In foetal and adolescent skeleton, type X is a transient intermediate in cartilage which is replaced by bone (Schmid and Linsenmayer, 1987). In adult, type X persists in the zone of calcified cartilage which separates the hyaline cartilage from the subchondral bone. Rotary shadowing of type X shows a rod-like domain and a globular domain at one end of the molecule (Figure 1.1), and rotary shadowed specimens appear to associate via their globular ends, but it is not known whether this occurs in vivo. The function and aggregated form of type X is not known. Although type X seems to be a quantitatively minor collagen, during the terminal stage of maturation it appears to be the major product of chondrocytes. The accumulation of type X precedes calcification of the cartilage, so two possible functions have been suggested for type X collagen; a) facilitation of removal of hypertrophic cartilage; b) formation of an ECM which is amenable to calcification.

Type XII is, as yet, uncharacterized, but it seems to resemble type IX collagen (Dublet and van der Rest, 1987; Gordon, Gerecke and Olsen, 1987; Dublet et al, 1988).

There are other proteins which contain collagen-like sequences: the complement component C1q, the pulmonary surfactant protein, acetylcholinesterase and a mannose-binding protein of liver (see Kühn, 1987, for references).

1.3 Type I Procollagen

1.3.1 Structure of the Type I Procollagen Molecule

Type I collagen is synthesized and secreted as a

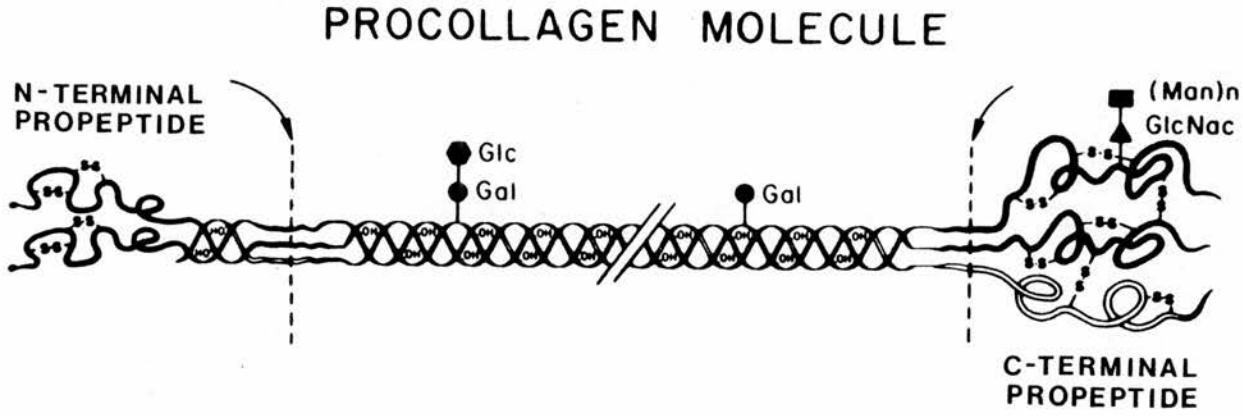

Figure 1.2 Representation of the procollagen molecule showing the N-propeptide, N-terminal telopeptide, triple helix, C-terminal telopeptide and C-propeptide domains (from Hulmes *et al*, in press, with permission).

precursor, procollagen, with extensions at either end of a central triple helical domain (Figure 1.2; Fessler and Fessler, 1978). These extensions are referred to as the amino (or N) -terminal and carboxyl (or C) -terminal propeptides, respectively, and these must be removed enzymatically before fibril formation can occur (Section 1.6).

The central domain of type I procollagen consists of three α-chains wound around each other in a triple helical conformation. The triple-helical domain is 300 nm long, as measured in the electron microscope by rotary shadowing. At either end of the molecule are short non triple helical regions which are known as telopeptides. The N-terminal telopeptide is 16-18 amino acids long, and the C-terminal telopeptide is 25 amino acids long. The type I collagen molecule consists of two α1 chains and one α2 chain, classified as such on the basis of genetic differences and differences in the pattern of cleavage of the two types of α chain by cyanogen bromide.

Within the triple helical structure the three helical chains are coiled about a central axis, so that the axis of each chain follows a right-handed path, while the overall helical symmetry is left-handed, with 10 units in three turns and a pitch of approximately 3 nm (for a review, see Ramachandran and Ramakrishnan, 1976). From packing considerations, and from sequence data, every third residue must be glycine since within the triple helix there is no space for a Cβ atom attached to a Cα (Figure 1.3). This helical conformation is stabilized by the presence of proline, as the 5-membered ring is rigid, and does not permit rotation about the N-Cα bond. The side chains of positions X and Y protrude from the chain, and thus positions X and Y can accommodate a variety of residues. The presence of 4-hydroxyproline stabilizes the structure by forming extra hydrogen bonds and contributes significantly to its thermal stability (Berg and Prockop, 1973). Within the triple helix the axial residue-to-

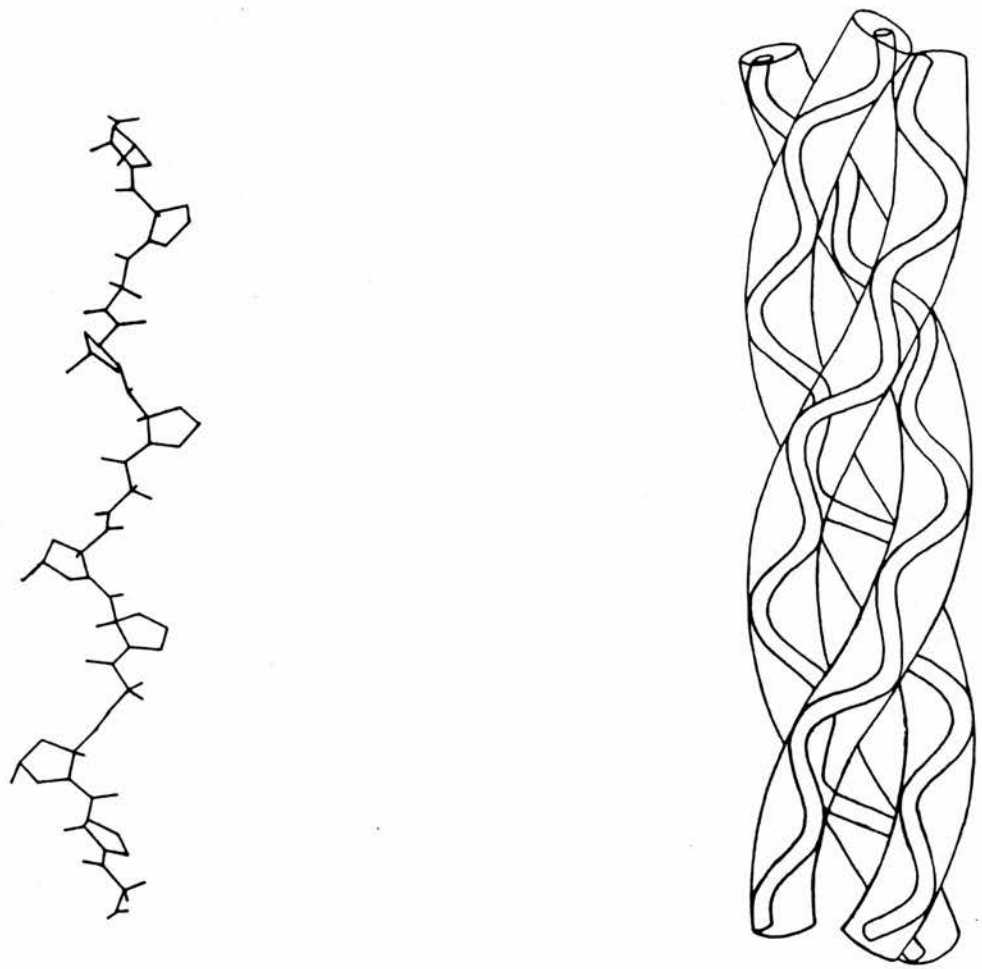

Figure 1.3 The collagen molecule. The left hand diagram shows a single α chain of the triple helix represented as gly-pro-hypro (adapted from Creighton, 1984); the right hand diagram shows how three such left-handed helices are given a right-handed twist to form a three-fold superhelix (adapted from Dickerson and Geis, 1969).

residue spacing is 0.286 nm (e.g. Chapman, 1984; Chapman and Hulmes, 1984). The conformation of the telopeptides is less certain, but they are thought to be more axially condensed than in the triple helix (Chapman and Hulmes, 1984; Wess, personal communication).

Conformational studies of the N-propeptide of type I procollagen using two related peptide fragments revealed that the N-propeptide differs in conformation from the triple helical region of the collagen molecule (Engel et al, 1977). There are three different domains of the N-propeptide; a large non triple helical region at the N-terminus of the proα1 chain which appears to have a rigid and ordered configuration as shown by circular dichroism (CD) studies and could be β structure; a short triple helical domain and a short non triple helical region which connects with the telopeptide, and thus the triple helix. There are thought to be five intrachain disulphide bridges. Interchain disulphide bonds are not present in the N-propeptide (e.g. Kivirikko and Myllyla, 1982). The high ratio of frictional coefficients f/f_{min} indicates that the large non triple helical domain (known as Col 1) has an elongated shape in solution with an axial ratio of 10:1. Engel et al (1977) calculated a length of 14 nm for this Col 1 fragment using this value of the axial ratio and a partial specific volume of 0.698 ml/g calculated from the amino acid sequence. The length of the triple-helical domain could be calculated from the amino acid sequence using a residue-to-residue spacing of 0.286 nm, giving a value for the length of the N-propeptide as 30 nm. The large N-terminal globular domain is not present in the proα2(I) chain, with the result that this chain has a smaller molecular weight. The N-propeptide of calf procollagen contains 138 residues (60 in the proα2(I) chain) and the sequence confirms the presence of a short collagenous region in the N-propeptide (Hörlein et al, 1979). The N-propeptide of the chick proα1(I) chain lacks the first seven residues of the calf proα1(I) chain. A

possible role for the cleaved N-propeptide has been suggested in feedback regulation of collagen synthesis (Wiestner et al, 1979; Section 1.5).

The C-propeptide of type I procollagen contains 8 cysteine residues, 6 of which participate in intrachain disulphide bonds. The remaining two cysteines form an interchain disulphide bridge (Olsen, Hoffmann and Prockop, 1976) which is important for the stabilization of the three chains (Kühn, 1987). The C-propeptide contains 246 residues in the proα1(I) chain and 245 in the proα2(I) chain (Miller, 1985). The sequences of the C-propeptides in the procollagen chains proα1(I), proα2(I), proα1(III) and proα2(V) are 53% homologous (Dion and Myers, 1987), and with no sequence similarities to the C-propeptide domain of type IV procollagen. The C-propeptides are likely to direct the association between the three pro α chains and to serve as an initiation site for triple helix formation (Kivirikko and Myllyla, 1984).

As with the N-propeptide, the C-propeptide of type I procollagen may have a role in feedback regulation of collagen synthesis (Aycock et al, 1986; Wu, Donovan and Wu, 1986). The C-propeptide of type II procollagen has been previously identified as the protein chondrocalcin, which may form a nucleation site for mineralization (Poole et al, 1984). The possible role of the procollagen propeptides in the regulation of collagen fibril formation is discussed below (Section 1.6.4.4).

1.3.2 Biosynthesis of Type I Procollagen

Collagen synthesis is characterized by a large number of post-translational modifications (Table 1.2), several of which are unique to collagen and a few other proteins with collagen-like sequences. This section will refer only to type I biosynthesis unless stated otherwise.

A brief overview of type I collagen synthesis will be

Biosynthetic Step	Biological Function
Intracellular Modifications	
Removal of signal sequence	Membrane translocation
Proline 3-hydroxylation	Unknown
Proline 4-hydroxylation	Triple helix formation
Lysine Hydroxylation	O-glycosylation of hydroxylysyl residues; cross-link formation
O-Glycosylation of hydroxylysyl residues	Unknown; possible role in fibril formation
N-Glycosylation of asparaginyl residues	Unknown
Chain association and disulphide bonding	Triple helix formation
Triple Helix Formation	Functional protein
Extracellular Modifications	
Removal of N-propeptide	Normal fibril morphology
Removal of C-propeptide	Fibril formation
Ordered aggregation	Formation of fibrils
Cross-link formation	Formation of stable fibrils

Table 1.2 Post-translational modifications in the synthesis of type I procollagen. It should be remembered that in other collagen types not all of the extracellular modifications occur, and that other collagen types may form non-fibrillar aggregates; also that sulphation of tyrosine residues occurs in type V procollagen. (Adapted from Kivirikko and Myllyla, 1985).

given here. However, it is not possible to cite all the original contributions in this field and therefore only a few selected references are noted. For a fuller treatment of the subject and for more detailed references the reader is referred to the reviews by Fessler and Fessler (1978); Prockop et al (1979); Heathcote and Grant (1980) and Kivirikko and Myllyla (1982, 1984).

1.3.2.1 Synthesis of Preprocollagen

Collagen genes are transcribed from DNA to mRNA in the way described for other eukaryotic proteins (for a review of collagen genes, see review in Cheah and Grant, 1982; Cheah, 1985). The mRNA is translated on membrane bound ribosomes (Diegelmann, Bernstein and Peterkofsky, 1973; Kivirikko and Myllyla, 1984). Procollagen is synthesized as a precursor, preprocollagen. This precursor has an amino-terminal extension which has a relatively hydrophobic sequence (Graves et al, 1979, 1981; Palmiter et al, 1981). The molecular weights of the pre sequence of preprocollagen have been reported as 5,500 daltons (Palmiter et al, 1981) or 10,000 daltons (Graves et al, 1979). Despite the difference in molecular weight of the pre sequence obtained by the two groups, it is clear that the leader sequence of procollagen is much larger than the signal sequences of other secretory proteins.

1.3.2.2 Cleavage of the signal sequence

Signal sequence cleavage has been reviewed by Walter, Gilmore and Blobel (1984) and Walter and Lingappa (1986) and the reader is referred to these articles for a fuller treatment of this aspect of protein maturation.

The signal sequence interacts with cytoplasmic receptors (signal recognition particle, SRP). SRP then seems to direct the nascent chain to the SRP receptor, which is an integral membrane protein of the rough endoplasmic

reticulum (rough ER), and this allows the nascent chain to be translocated across the rough ER membrane, although how this is achieved is not clear. The signal peptide is then cleaved by a signal peptidase. That preprocollagen is translocated by this system was demonstrated by Palmiter et al (1979). When partially purified procollagen mRNA was translated in the presence of dog microsomal membranes, the major products migrated more rapidly on SDS-PAGE than control preparations, indicating that the pre sequence had been cleaved by a signal peptidase activity.

1.3.2.3 Proline and Lysine Hydroxylation

The post-translational modification reactions are summarized in Table 1.2. The hydroxylating activities are associated with the ER (Diegelmann, Bernstein and Peterkofsky, 1973; Harwood, Grant and Jackson, 1974; Kirk, Evans and Veis, 1987). Three enzymes carry out the hydroxylation reactions: prolyl 4-hydroxylase (EC 1.14.11.2), prolyl 3-hydroxylase (EC 1.14.11.?) and lysyl hydroxylase (EC 1.14.11.4). Very little 3-hypro is found in types I, II, and III and its physiological role is not known. Prolyl 4-hydroxylase hydroxylates pro residues at the 4-position in the sequence gly-X-pro (for detailed reviews, see Kivirikko and Myllyla, 1982, 1984; Kivirikko and Majamaa, 1985). Ascorbate, Fe^{2+} and α-ketoglutarate are necessary cofactors for the reaction. Lysyl hydroxylase acts on lys residues in the Y position in the gly-X-Y sequence and has the same reaction mechanism and cofactors as prolyl 4-hydroxylase. Further hydroxylation is prevented by triple helix formation. 4-Hydroxylation of pro residues results in thermal stabilization of the triple helix (Berg and Prockop, 1973). Thus at body temperatures, unhydroxylated α chains cannot fold into a triple helix. Currently there is much interest in developing compounds which can inhibit prolyl 4-hydroxylase, as a strategy for preventing the excessive

deposition of collagen which occurs in fibrotic conditions (Uitto, Tan and Ryhanen, 1982; Kivirikko and Myllyla, 1985; Kivirikko and Majamaa, 1985).

Hydroxylation of lys residues is necessary for the addition of O-linked sugar units and for the stability of intermolecular cross-links (see Table 1.2)

1.3.2.4 Glycosylation of Hydroxylysyl Residues

The O-linked sugars are found in the form galactosyl-O-lys and glucosylgalactosyl-O-lys. The formation of the sugars is catalyzed by two specific enzymes, peptidyl hydroxylysylgalactosyl transferase (EC 2.4.1.50) and peptidyl galactosylhydroxylysylglucosyl transferase (EC 2.4.9.66). Glycosylation seems to continue until triple helix formation is complete. Analysis of subcellular fractions showed that glycosylation begins in the ER, and thus begins on the nascent chains. Glycosylation also takes place throughout the smooth ER (Harwood, Grant and Jackson, 1975).

1.3.2.5 Glycosylation of Asparaginyl Residues

The C-propeptide of the chick type I procollagen proα1 chain contains an asn residue in the sequence asn-val-thr, which appears to contain oligosaccharide units (Clark, 1979). The oligosaccharide consists of N-acetylglucosamine and mannose. The biosynthesis of asparaginyl N-linked units is initiated by the en bloc transfer of a preformed oligosacharide from a lipid carrier. This oligosaccharide is processed further within the rough ER and smooth ER and Golgi apparatus (Rothman, 1985). The glycosylation of procollagen has likewise been shown to involve a lipid intermediate (for references, see Kivirikko and Myllyla, 1982). The function of the asn-linked units is unknown.

1.3.2.6 Chain Association, Disulphide Bonding and Triple Helix Formation

In type I procollagen, interchain disulphide bonds form only between the C-propeptides (Harwood et al, 1977). Disulphide bond formation starts 7 minutes after synthesis of procollagen begins, and is virtually complete after 13 minutes (Schofield, Uitto and Prockop, 1974). Thus, disulphide bond formation occurs after assembly of amino acids into proα chains and after their release from ribosomes. In the presence of α,α'-dipyridyl, an inhibitor of proline hydroxylation, disulphide bond formation still takes place, so a helical structure is not necessary for disulphide bond formation or chain association (Schofield, Uitto and Prockop, 1974; Uitto and Prockop, 1974; Harwood et al, 1977). Disulphide bonding and triple helix formation occurred at about the same time (Schofield, Uitto and Prockop, 1974). The enzyme protein disulphide isomerase (PDI) is thought to play a role in disulphide bond formation in procollagen (Koivu and Myllyla, 1987). The PDI activity has been shown to correlate with the activity of prolyl 4-hydroxylase (Kivirikko and Myllyla, 1985) and recently the PDI activity has been shown to exist in the β subunit of prolyl 4-hydroxylase (Koivu et al, 1987). Subcellular fractionation has shown that disulphide bonding and triple helix formation take place in both fractions of the ER (Harwood et al, 1977).

Hydroxylation is not required for correct chain association as unhydroxylated procollagen can also associate in the 2:1 ratio (Harwood et al, 1977). Association of the three chains seems to occur simultaneously, as no dimeric intermediates are seen (Harwood et al, 1977).

1.3.2.7 Transport and Secretion of Procollagen

Autoradiography indicates a route of transport through

the cell from the rough ER through the Golgi apparatus and via secretory vesicles to the cell surface (Weinstock and Leblond, 1974; Morris et al, 1975; Marchi and Leblond, 1983). Ferritin-conjugated antibodies against procollagen labelled the ER and Golgi (Olsen and Prockop, 1974). This route was confirmed in biochemical studies by Harwood, Grant and Jackson (1976) using pulse-chase techniques and subcellular fractionation at various chase times. Tendon procollagen was secreted 20 minutes after the initiation of protein synthesis, while cartilage procollagen was secreted 35 minutes after the start of protein synthesis, which possibly reflects the extra glycosylation of type II procollagen. Embryonic chick calvarial procollagen (also type I) was secreted 30 minutes after the start of protein synthesis (Weinstock and Leblond, 1974), while embryonic chick corneal type I procollagen was secreted 25 minutes after synthesis started (Kao, Mai and Chou, 1982). Procollagen appears to be secreted in a biphasic process (Kao, Berg and Prockop, 1977; Kao, Prockop and Berg, 1979). Secretion can be inhibited using α,α'-dipyridyl, so that triple helix formation is necessary for secretion.

Several workers (Trelstad, 1971; Weinstock and Leblond, 1974; Bruns et al, 1979; Trelstad and Hayashi, 1979; Cho and Garant, 1981; Marchi and Leblond, 1983) have observed structures in secretory vesicles that seem to correspond to bundles of procollagen in non-staggered array, so it has been suggested that procollagen is secreted in an aggregated form. Centrosymmetric structures were observed in secretory vacuoles of the odontoblast process and in predentin (Weinstock, 1977). Others have not observed such aggregates in secretory vacuoles of human fibroblasts (Phelps, Martinez-Hernandez and Goldberg, 1985).

1.3.2.8 Removal of Propeptides

Kinetic data indicate that the propeptides are removed coincident with, or subsequent to, secretion (Morris et

al, 1975). Pulse labelling studies showed that propeptide removal occurred at incubation times longer than 30 minutes after the administration of a radioactive label, at the same time that secretion was occurring, as shown by autoradiography. Processing of type III procollagen is much slower than processing of type I procollagen, and therefore propeptide removal also takes place after secretion in type III procollagen (Fessler, Timpl and Fessler, 1981). Incubating matrix-free chick embryo tendon cells with colchicine (1 μM) prevents secretion of procollagen and blocks the removal of the propeptides (Fessler and Fessler, 1978; Uitto, Allan and Polak, 1979). Pulse chase studies and autoradiographic techniques showed that propeptide removal from type II procollagen did not occur until after secretion in chick embryo sterna (Uitto, Allan and Polak, 1979). Incubation of intact sterna with compounds which inhibit hydroxylation delayed secretion and prevented processing of type II procollagen (Uitto, Allan and Polak, 1979). Addition of the compounds after secretion had no effect on processing. Thus, secretion is a prerequisite for processing to occur.

1.3.2.9 Early Work on Processing Enzymes

Conversion of type II procollagen to collagen secreted by matrix-free chick sternal cells continued even after the cells were removed by centrifugation (Uitto, Allan and Polak, 1979). Hence, the enzymes are secreted into the medium and can function extracellularly without being associated with the cells.

Goldberg, Taubman and Radin (1975) using a crude cell extract which contained "procollagen peptidase" activity demonstrated that cleavage was specific and not pepsin-like, as cleavage with the procollagen peptidase produced only one fragment. Random cleavage would have produced several fragments. Uitto and Lichtenstein (1976) showed that intact 17 day chick embryo tendons contained enzyme

activities which could remove both propeptides from procollagen. The two activities were shown to be separate, because medium from matrix-free cells could remove the N-propeptide, i.e. having N-proteinase activity but the C-propeptide-cleaving activity (C-proteinase) was no longer present. This indicated that the C-proteinase is removed or inactivated during the isolation of matrix-free cells. Leung et al (1979) separated the two enzyme activities using DEAE-cellulose chromatography and showed that the two enzymes differed in charge properties, i.e. there were two separate peaks of activity. Incubation of pC-collagen (procollagen without the N-propeptide) or collagen with the two proteinases and lysyl oxidase (Section 1.3.2.12) revealed that the telopeptides remained intact after peptidase action, because the cross-links were formed normally.

The properties of the procollagen processing enzymes have been reviewed by Peltonen, Halila and Ryhanen (1985). For the purposes of this thesis, pC-collagen is defined as procollagen from which the N-propeptide has been removed, and pN-collagen is defined as procollagen from which the C-propeptide has been removed (Figure 1.4).

1.3.2.10 Procollagen N-Proteinase

Type I procollagen N-proteinase has been purified from calf leg tendons (Kohn et al, 1974); leg tendons of 17-day chick embryos (Tuderman, Kivirikko and Prockop, 1978; Tuderman and Prockop, 1982; Prockop and Tuderman, 1982) and from whole embryos (Tanzawa, Berger and Prockop, 1985). Most recently, procollagen N-proteinase has been extracted from the leg tendons of 17-day chick embryo tendons using a modification of the method of Tuderman and Prockop (1982) (Hojima, unpublished; Hulmes, personal communication). The molecular weight of the enzyme as determined by gel filtration has been given as 260,000 daltons (Tuderman, Kivirikko and Prockop, 1978; Tuderman

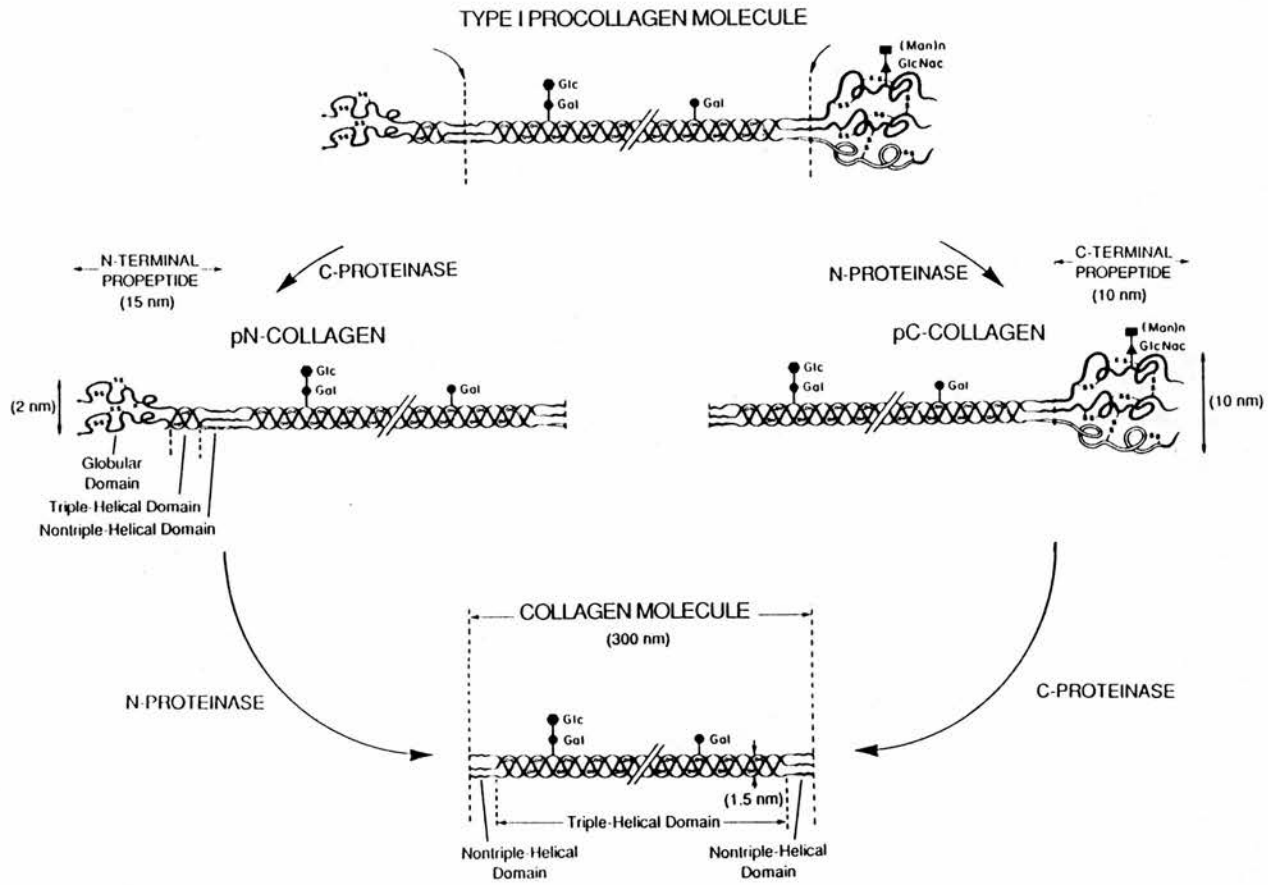

Figure 1.4 Pathways of processing of procollagen by procollagen N-proteinase and procollagen C-proteinase (from Hulmes et al, in press, with permission).

and Prockop, 1982) and 320,000 daltons (Tanzawa, Berger and Prockop, 1985). The reason for the discrepancy is not known.

Calcium ions are a cofactor for the enzyme, 5 mM Ca^{2+} being most effective (Uitto, 1977; Tuderman, Kivirikko and Prockop, 1978; Tuderman and Prockop, 1982; Tanzawa, Berger and Prockop, 1985). Ca^{2+} also appears to stabilize the enzyme to heat. Disodium ethylenediaminetetraacetate (EDTA) inhibits the enzyme activity, which further suggests a requirement for calcium ions. Tuderman and Prockop (1982) have suggested that a second metal ion may be necessary for activity, since preincubation of the enzyme with EDTA for 10 minutes and then adding a 10-fold molar excess of Ca^{2+} failed to fully restore the activity. However, no second metal ion has been found.

The K_m for the reaction was found to be 1 μM by Tuderman, Kivirikko and Prockop (1978), but this was not determined accurately. When pN-collagen was used as a substrate for the enzyme, the K_m was in the region of 0.3-0.5 μM (Tuderman and Prockop, 1982).

The N-proteinase only cleaves a substrate in the native triple helical conformation (Tuderman, Kivirikko and Prockop, 1978; Tuderman and Prockop, 1982; Tanzawa, Berger and Prockop, 1985). Tuderman and Prockop (1982) showed that pN-collagen heated to 55°C for 10 minutes did not act as a substrate for the enzyme. Incubating chick and human type I procollagens with the enzyme at temperatures ranging from 39°C to 45°C demonstrated that each of the procollagens became resistant to cleavage at about the same temperature that the helix-coil transition took place, although the enzyme itself was stable at temperatures ranging from 44 - 48°C (Tanzawa, Berger and Prockop, 1985).

The tendon-derived N-proteinase cleaves type II procollagen (Tuderman, Kivirikko and Prockop, 1978; Tanzawa, Berger and Prockop, 1985) but not type III or type IV procollagen (Tuderman and Prockop, 1982; Tanzawa,

Berger and Prockop, 1985). A separate N-proteinase activity which cleaves type III procollagen has been isolated from calf tendon fibroblasts (Nusgens et al, 1980), bovine aortic smooth muscle cells (Halila and Peltonen, 1984) and from human placenta (Halila and Peltonen, 1986). The enzyme is specific for type III procollagen. The K_m determined by Nusgens et al (1980) was 0.3 μM; that determined by Halila and Peltonen (1984) was 0.76 μM; and the K_m for the human enzyme was 2 μM (Halila and Peltonen, 1986). The two N-proteinases are different enzymes as they have a) different substrate specificities; b) different molecular weights; c) different behaviour towards inhibitors, although they share several common features (Nusgens et al, 1980); d) antibodies against the type I enzyme do not affect the type III enzyme, or alter the activity when added to the reaction mixture (Nusgens et al, 1980); e) differential affinities for Concanavalin A-Sepharose (Nusgens et al, 1980); f) different K_ms. The requirement for a different enzyme might be explained by the fact that the N-propeptides have different amino acid compositions (Nusgens et al, 1980). Halila and Peltonen (1984) suggest that substrate specificity may be important as types I and III form different kinds of collagen fibrils. In fact, the presence of two separate enzyme activities is of great interest, as it is now known that types I and III can coexist in the same fibril (see Section 1.6.4.3; Keene et al, 1987).

The site of cleavage of procollagen by N-proteinase is at the bond pro 139-gln 140 (Hörlein et al, 1979; Kivirikko and Myllyla, 1984). The susceptible bond is not surrounded by glycine residues. There are several hydrophobic amino acids in the vicinity (Hörlein et al, 1979). Cleavage at the pro-gln bond results in cyclization of the glutamyl residue to form pyroglutamic acid (Heathcote and Grant, 1980). Wozney et al (1981) identified a presumptive proα2(I) cleavage site: asn-phe-ala-ala-gln, with cleavage at ala-gln.

Morikawa, Tuderman and Prockop (1980) synthesized peptides ranging in length from 4-13 amino acids. The sequence of the peptides corresponded to the amino acid sequence around the cleavage site of the chick proα1(I) chain (Hörlein et al, 1979; Pesciotta et al, 1980). The peptides did not act as substrates for the N-proteinase, which was expected, because of the enzyme's requirement for the substrate to be in a native conformation (Tuderman, Kivirikko and Prockop, 1978; Tuderman and Prockop, 1982; Halila and Peltonen, 1984; Tanzawa, Berger and Prockop, 1985; Halila and Peltonen, 1986). Certain peptides acted as inhibitors of the N-proteinase activity (Morikawa, Tuderman and Prockop, 1980). The presence of a phenylalanyl residue in the sequence phe-ala-pro-gln was essential for inhibitory activity, as replacement of the phe by tyr, gly, lys, glu or D-phe abolished the inhibitory activity. Derivatives of ferrocenes acted as specific, reversible competitive inhibitors of the N-proteinase (Dombrowski, Sheats and Prockop, 1986).

Berger, Tanzawa and Prockop (1985) examined the sequential cleavage of the N-propeptide by the N-proteinase, by visualizing the stages of the cleavage using SDS-PAGE, fluorography and densitometry. The first step of the reaction appeared to be random cleavage of the proα1 or proα2 chains, so all the proα2 chains were cleaved before the proα1 chains.

1.3.2.11 Procollagen C-Proteinase

Procollagen C-proteinase was initially purified by Njieha et al (1982) from the calvaria of 17-day chick embryos and had a molecular weight of 80,000 daltons as determined by gel filtration. The C-proteinase was extensively purified from the organ culture of 17-day chick embryo tendons by Hojima, van der Rest and Prockop (1985). The molecular weight of the Hojima enzyme was 102,000 daltons (reducing SDS-PAGE), 97,000 daltons

(unreducing conditions on SDS-PAGE) and 110,000 daltons (gel filtration). Like the N-proteinase (see Section 1.3.2.10) the C-proteinase was shown to require calcium ions for optimal activity, at concentrations of Ca^{2+} of 5-10 mM (Hojima, van der Rest and Prockop, 1985). It is not apparent whether the enzyme isolated by Hojima et al is the same as the enzyme of Njieha et al, because of the different molecular weights and pattern of inhibition.

In contrast to the N-proteinase, the C-proteinase does not require the procollagen substrate to be in a native triple helical conformation, as it readily cleaved heat-denatured procollagen (Hojima, van der Rest and Prockop, 1985). The enzyme also cleaves types II and III procollagen at the same rate as type I procollagen (Njieha et al, 1982; Hojima, van der Rest and Prockop, 1985) and this may be the sole C-proteinase found in vivo, whereas different enzymes exist to remove the type I and type III N-propeptides, although there has also been a preliminary report of differential inhibition of type I and type III C-proteinase cleavage by lysine (Peltonen, Halila and Ryhanen, 1985).

Hojima and colleagues (1985) suggest that as the turnover number of the enzyme is relatively low (k_{cat} = 41 h^{-1}), and the initial concentration is 1 μg/tendon, the enzyme may be present in tissue as an inactive precursor, or readily inhibited by some endogenous component of connective tissues.

Procollagen C-proteinase has also been purified from mouse fibroblast cell culture (Kessler et al, 1986). The mouse enzyme shows the same catalytic properties as the chick enzyme. A 35,000 dalton enhancer of C-proteinase activity has been isolated from mouse fibroblast cultures (Adar et al, 1986). This enhancer is a glycoprotein capable of binding to the C-propeptide, and it increases the K_m and V_{max} of the reaction.

The site of cleavage of the proα1(I) and proα2(I) chains by the C-proteinase is at an ala-asp bond (Dickson et al,

1981; Fuller and Boedtker, 1981; Peltonen, Halila and Ryhanen, 1985). The C-proteinase isolated by Hojima et al (1985) specifically cleaved at this ala-asp bond.

Morris et al (1979) investigated the sequence of cleavage of the C-propeptide. The first cut was found to be random, but the second cut was made more frequently in the pC-collagenα2 (pCα2) chain than would be expected for random cleavage.

Njieha et al (1982) also carried out experiments to determine the order of cleavage. In experiments where the reaction was carried out so that only part of the substrate was cleaved, α1 chains were released prior to, and in greater proportion than α2 chains, which suggests that the proα1/pCα1 chain is cleaved first. A partial reaction was carried out and the products examined by 2-D gel electrophoresis. The major intermediates seen in the cleavage of pC-collagen to collagen are pCα1-pCα2-C-propeptide (C1) and pCα2-C1-C1, which indicates that the preferential order of cleavage is pCα1, pCα1, and then pCα2.

1.3.2.12 Ordered Aggregation and Cross-Link Formation

The collagen molecules which have been formed by removal of the propeptides assemble spontaneously in a specific manner to form fibrils; no enzyme is required to catalyze this step. If procollagen is secreted in the form of ordered aggregates, as suggested by Bruns et al (1979), then processing could give rise to structures that could be directly incorporated into fibrils (Hulmes, 1983). Fibril structure and assembly are discussed in sections 1.6.1, 1.6.2 and 1.6.3.

The initial step in cross-link formation is oxidative deamination of the ϵ-amino group of lysine and hydroxylysine to form allysine and hydroxyallysine. This step is catalyzed by lysyl oxidase (EC 1.4.3.13), a copper-requiring enzyme which requires molecular oxygen

(for references, see Kivirikko and Myllyla, 1984). Lysyl oxidase also catalyzes the first step in elastin cross-link formation (for a review, see Robins, 1982). After the initial oxidative deamination step, the next reaction is a spontaneous non-enzymic condensation of the aldehyde group with amino groups on adjacent molecules. Reaction of the aldehyde group of hydroxyallysine results in a more stable cross-link, because the Schiff base formed can undergo an Amadori rearrangement to a keto-imine. In tissues such as bone and cartilage, where there is a large amount of hydroxylysine, these keto-imine cross-links are the major component (Robins, 1982). The cross-linking amino acids have been identified after chemical reduction with tritiated sodium borohydride, which stabilizes them during acid hydrolysis and labels them with tritium for isolation. Glycosylated hydroxylysine can also form these cross-links.

The amount of these reducible cross-links decreases with age and this led to the hypothesis that the reducible cross-links are intermediates which are converted during maturation to a more stable, non-reducible form (for references, see Robins, 1982). A pyridinoline cross-link has been isolated from bovine Achilles tendon, and it was postulated that the compound could be derived from two hydroxylallysines and one hydroxylysine (for references, see Eyre, Paz and Gallop, 1984).

Cross-link formation has been shown to be necessary for tensile strength, as shown by induction of experimental lathyrism, and an X-chromosome linked form of cutis laxa in which lysyl oxidase is deficient, and also in animals with copper deficiency (for references, see Eyre, Paz and Gallop, 1984).

1.3.3 Degradation of Collagen

In the fibrillar state, collagen is resistant to proteolytic degradation and can only be degraded by

specific neutral metalloproteases, collagenases (for reviews, see Harris, Welgus and Krane, 1984; Jeffrey, 1986). Most collagenases only cleave collagen types I, II and III, and collagenases from different cellular sources vary in their substrate specificity. It has been reported that type X collagen can be cleaved by these collagenases, but other non-fibrillar collagens are not (references in Schmid and Linsenmayer, 1987). A separate collagenase cleaves type IV collagen (for references, see Harris, Welgus and Krane, 1984), and an enzyme from canine kidney epithelial cells was shown to specifically degrade type V collagen in sections of embryonic chick cornea (Fitch et al, 1988). The enzyme which cleaves the interstitial collagens does so at gly 775-ile 776 in the α1(I) chain (leu in α2(I) chain), has a Zn^{2+} ion at the active site and requires Ca^{2+} ions for maximal activity. Once cleavage has occurred, the two fragments produced (TC_a and TC_b) denature rapidly and become suitable substrates for a variety of other tissue metalloproteinases such as gelatinase and stromelysin. These fragments can either be phagocytosed and degraded by cysteine proteases at acid pH (cathepsins) in lysosomes, or can be further fragmented extracellularly resulting in small peptides and amino acids.

Collagenase from human skin is secreted as an inactive precursor, procollagenase, which exists in two forms of molecular weight 60,000 and 55,000 daltons. These are converted into active forms some 10,000 daltons lower in molecular weight, by several proteases (trypsin, cathepsin B, kallikrein, mast cell proteases and plasmin: see Harris, Welgus and Krane, 1984, for references), and by certain organomercurial compounds which alter the environment of the procollagenase to an active zymogen form and permit self-cleavage (Jeffrey, 1986). Procollagenase is also activated by stromelysin (Murphy et al, 1987).

Collagenase released into the extracellular matrix and

activated will degrade substrate until it is inhibited or inactivated. Thus, a careful system of control must operate, otherwise collagenolysis would proceed unchecked. Specific inhibitors (tissue inhibitors of metalloproteinase, TIMP) have been identified in cultures of tissue explants and connective tissue cells. These 30,000 dalton proteins are resistant to extremes of pH and temperature and form a complex with activated collagenase with very high affinity (K_i about 10^{-8} M), 2 orders of magnitude greater than the apparent affinity of collagenase for its substrates. This implies that large changes in enzyme activity could occur on relatively small changes in amount of either reagent (Jeffrey, 1986). Two other inhibitors of collagense which are found in serum are α_2-macroglobulin and β_1-anticollagenase (which cross-reacts immunologically with TIMP).

Procollagenase synthesis was stimulated and TIMP synthesis was coordinately decreased in rabbit uterine cervical fibroblasts by human recombinant interleukin-1α (Ito et al, 1988).

1.4 Processing of Genetically Distinct Collagen Types

For the purposes of this thesis, processing is defined as removal of one or both of the propeptides by the action of specific N- and C-proteinases.

The interstitial procollagens types I, II and III are all processed enzymatically to collagen after secretion (see Section 1.3.2.8) (Figure 1.4). Type IV procollagen is not processed (for references, see Kivirikko and Myllyla, 1982, 1984, and Glanville, 1987). Of the other collagen types, types V and XI are processed to lower molecular weight forms (references in Fessler and Fessler, 1987; Eyre and Wu, 1987). Type VI is not thought to be processed in cell culture even at very long chase times (Colombatti and Bonaldo, 1987), although this contrasts with a previous report in which processing was detected (Trüeb

and Winterhalter, 1986). Type VII is not processed (Burgeson, 1987). Sage, Trüeb and Bornstein (1983) examined the biosynthesis of type VIII collagen using pulse-chase techniques, and did not observe processing of this collagen to lower molecular weight forms. Type IX collagen is not processed (Grant et al, 1985; van der Rest and Mayne, 1987). Type X procollagen has been detected in a lower molecular weight form by Grant et al (1985), but not by other groups (for references, see Schmid and Linsenmayer, 1987). Ayad, Kwan and Grant (1987) suggest that the failure of other groups to detect processed type X collagen may be due to differences in salt concentration used to precipitate out the type X.

The processing of type V procollagens shows some unusual features (Fessler, Shigaki and Fessler, 1985; Fessler and Fessler, 1987). The procollagens V retain some non-collagenous portions 22 hr after a 2 hr pulse period. Procollagen V of chick blood vessels is cleaved via a so-called "p-collagen V" intermediate and is converted only slowly to α-collagen V, similar to the processing of type III procollagen (Fessler, Timpl and Fessler, 1981). The conversion of procollagen V to p-collagen V appears to proceed via removal of the C-propeptide (Fessler, Shigaki and Fessler, 1985). During the removal of the C-propeptide, a non-collagenous peptide (P, 35,000 daltons) becomes covalently attached to the molecule. Procollagen V prepared from chick embryo blood vessels was incubated with procollagen peptidases derived from chick tendon culture medium (Leung et al, 1979) and processing of the procollagen V occurred. SDS-PAGE showed that this cleavage was specific, as these processed components migrated at the same position as chick tendon fibroblast processed procollagen V. Chinese hamster lung cells, which did not undergo type V processing in culture, were processed by the addition of the procollagen peptidase activities to p-collagen V (Fessler, Robinson and Fessler, 1981). Interestingly, although the nature of the type V cleaving

enzymes is not yet clear. 50 mM arginine, which inhibits C-propeptide removal in type V collagen in lung cell and tendon cultures (Fessler, Robinson and Fessler, 1981) and in chick embryo crop (Kumamoto and Fessler, 1981). Sulphated proα1(V) chains were processed more rapidly than sulphated proα1'(V) chains and pα2(V) was processed more rapidly than sulphated pα1(V) chains (Fessler et al, 1986; Fessler and Fessler, 1987).

1.4.1 Pathways of Procollagen Processing

The order of processing of type I procollagen has been extensively studied in cell culture systems and in intact tissues.

The order of removal of the propeptides has been investigated by pulse-labelling and pulse-chase techniques in various tissues. Labelling of intact tendons and DEAE-cellulose chromatography revealed the presence of a pC-collagen intermediate, which seemed to indicate that the initial step is removal of the N-propeptide (Uitto and Lichtenstein, 1976; Leung et al, 1979). Leung et al (1979) suggested that the apparently sequential removal of propeptides is due to sequential exposure of the substrate to the enzymes. In intact calvaria, N-propeptide cleavage was also thought to precede C-propeptide cleavage (Fessler, Morris and Fessler, 1975; Morris et al, 1975; Davidson, McEneany and Bornstein, 1975; 1977). Sodek and Limeback (1979) showed that in cleavage of type I procollagen in periodontal ligament, pC-collagen was mainly present, although some pN-collagen was seen.

In tendon cell culture, pC-collagen was the observed intermediate (Uitto, Lichtenstein and Bauer, 1976; Leung et al, 1979). In guinea pig fibroblast culture, the N-propeptide seemed to be removed first, but in about 6% of the procollagen, the C-propeptide seemed to be removed first, as small amounts of pN-collagen were seen (Sonohara et al, 1981). This order of processing was also observed

in bovine aortic smooth muscle cells (Gerstenfeld et al, 1984). Monkey periodontal ligament cultures seemed to process the type I procollagen via pC-collagen, although in rat periodontal ligament cultures, pN-collagen was also observed (Limeback and Sodek, 1979; Sodek and Limeback, 1979). In the presence of 5% polyethylene glycol, both pN- and pC-collagens were present as processing intermediates, suggesting a more random order of processing (Bateman et al, 1986). Pulse-chase experiments indicated that the C-propeptide was removed first, with an initial transient accumulation of pN-collagen (Bateman et al, 1986).

Pulse-chase experiments on intact 17-day embryonic chick sterna showed the presence of both intermediates on SDS-PAGE, indicating a random order of removal of propeptides from type II procollagen (Uitto, 1977; Uitto, Allan and Polak, 1979). However, Ryhanen et al (1982) did not observe the presence of type II pN-collagen.

Pulse-labelling chick blood vessels indicated that type III procollagen was converted to collagen via a pN-collagen intermediate (Fessler and Fessler, 1979; Fessler, Timpl and Fessler, 1981). Processing of type III procollagen appears to be much slower than processing of type I procollagen, as processing of type I procollagen was complete after 2 hours, whereas type III procollagen was converted to pN-collagen III after three hours, with only slow conversion to collagen III (80% complete by 20 hours) (Fessler, Timpl, and Fessler, 1981). Sodek and Limeback (1979) and Limeback and Sodek (1979) compared type I and type III procollagen conversion in the periodontal tissues in the rat. The intermediate suggested by these workers was not pN-collagen type III, but pC-collagen type III. They also observed that the intermediate persisted for long times and that even after 24 hours there was little conversion to type III collagen. The presence of a pC-collagen type III intermediate in bovine aortic smooth muscle cell cultures was observed by Gerstenfeld et al (1984).

In osteoblast culture, the type I procollagen appeared to be processed via pC-collagen (Gerstenfeld et al, 1988). The extent of processing apparently increased with the number of days in culture, and the total amount of collagen in the extracellular matrix also increased. Calcification was also occurring, demonstrating the relationship between matrix maturation and procollagen processing.

The C-propeptide appears to be removed first from type V procollagen, and a non-collagenous peptide P becomes attached to the intermediate (p-collagen). Cleavage of the N-propeptide seems to take place very slowly, and the p-intermediate persists even after long chase times in culture (Fessler, Shigaki and Fessler, 1985; Fessler and Fessler, 1987).

Many inherited diseases of collagen can involve processing defects (see Section 1.7). The order of propeptide removal is thought to be important in the regulation of fibril diameter (see Section 1.6.4.4).

1.5 Regulation of Collagen Synthesis

There are three levels at which collagen biosynthesis can be regulated: a) regulation of the genetic type of collagen which is synthesized; b) regulation of the amount of a particular type of collagen which is synthesized; and c) regulation of the extent of post-translational modifications (Kivirikko and Myllyla, 1984, 1985).

Freshly isolated tendon fibroblasts seem to produce only type I collagen (Dehm and Prockop, 1971) and freshly isolated chondrocytes produce only type II collagen. However, some cells can synthesize two collagen types simultaneously (Conrad, Dessau and von der Mark, 1980).

The amount of a particular collagen type synthesized and the level of post-translational modifications can be influenced by growth, development and ageing (Schofield,

Freeman and Jackson, 1971: Linsenmayer, Fitch and Mayne, 1984).

Genetic regulatory elements have been described, including a 274 base pair sequence in the first intron of the human α(I) gene which is inhibitory (Bornstein et al, 1987). This appears to flanked by sequences that modify this inhibitory effect and confer a net positive effect on the sequence (Bornstein and McKay, 1988). An 800 base pair sequence in the first intron of the rat α1(II) gene which acts as an enhancer (Horton et al, 1987), operates when placed either upstream or downstream. This enhancer becomes active during differentiation of limb bud cells into chondrocytes and enhancer activity is lost in chondrocytes treated with retinoic acid, which suppresses chondrogenesis.

Regulation of collagen biosynthesis can be affected by the amounts of modifying enzymes (Kivirikko and Myllyla, 1984, 1985). The levels of prolyl 4-hydroxylase, prolyl 3-hydroxylase, lysyl hydroxylase, galactosyl hydroxylysine transferase and glucosylgalactosyltransferase are known to vary amongst different cell types and tissues and in the same cell type or tissue in many experimental and clinical conditions (for references, see Kivirikko and Myllyla, 1984, 1985), e.g. during prednisolone treatment (Risteli, 1977). This may explain the differences in levels of post-translational modification between different tissues, as there is very little evidence for the presence of isozymes. The rate of triple helix formation is the same for tendon and corneal type I collagen (Kao et al, 1983), but could be different for genetically different types of collagen (Kivirikko and Myllyla, 1984).

Little is known about control of extracellular events. Lysyl oxidase and procollagen N-proteinase activities are known to vary in the culture medium of human fibroblasts, with maximum values at the stationary phase of growth occurring at about the same time as prolyl 4-hydroxylase activity (see Kivirikko and Risteli, 1976). Regulation of

the activity of the propeptide processing enzymes could also be affected by the ratio of enzymes to the substrate, and by factors which modify the concentrations of substrate and enzymes. The rate of procollagen synthesis affects the ratio of the enzymes to their substrates and thus changes in the rates of procollagen synthesis could affect the extent to which the chains are modified.

The N-propeptides of type I and type III procollagen have been shown to inhibit the synthesis of their respective collagen types by negative feedback in calf skin fibroblasts (Wiestner et al, 1979). The type III N-propeptide also affected the synthesis of type I procollagen to the same extent as did the type I N-propeptide. The observed inhibitory effects were not due to enhanced breakdown, as dialysis of the samples did not reveal any change in the amount of ^{3}H-hypro in the dialyzate. However, the biosynthetic step affected by the N-propeptides in these experiments was not known.

A synthetic peptide derived from the C-propeptide of the proα1(I) chain of human procollagen was shown to inhibit collagen synthesis in human lung fibroblasts (Aycock et al, 1986). The presence of the synthetic peptide for 24 hr reduced the level of collagen synthesis to 58% of the control level. The C-propeptide fragment did not appear to alter the steady-state levels of procollagen (and fibronectin) mRNAs. In contrast, Wu, Donovan and Wu (1986) demonstrated that in human lung fibroblasts the N-propeptide and the C-propeptide reduced the levels of procollagen mRNA. However, the two propeptides appeared to act differently. At low concentrations, although the N-propeptide inhibited procollagen mRNA levels, the inhibition of collagen synthesis was proportionately greater. This could be due to a mainly translational effect. There was a close correlation between inhibition of collagen synthesis and procollagen mRNA levels for the C-propeptide, suggesting that the C-propeptide was acting at a pre-translational level.

Paglia et al (1979) demonstrated an inhibitory effect of the types I and III N-propeptides on the translation of type I procollagen and by the type II N-propeptide on type II collagen synthesis (Paglia et al, 1981). However, the effect on type II synthesis was shown only in a reticulocyte lysate, and could not be observed in chondrocytes. The possible mechanism of inhibition of synthesis by propeptides could either involve uptake of the propeptides by the cell, or action of the propeptides by a receptor-mediated second messenger effect. Moen, Rowe and Palmiter (1979) examined the regulation of procollagen synthesis during the development of calvarium in 10-17 day old chick embryos. The increase in collagen synthesis during this period is due to a decrease in the rate of non-collagenous protein synthesis.

Tendon, skin and smooth muscle cells were shown to contain identical amounts of type I procollagen mRNAs, although skin and smooth muscle synthesize much less type I procollagen than tendon cells (Focht and Adams, 1984). This indicates that these mRNAs have inherently different "translatabilities", but differences in the mRNA structure were not apparent. In chondrocytes, there are small, but significant levels of the mRNAs necessary for type I procollagen synthesis, although no type I synthesis was seen. Investigation of the sizes of the proα1(I) and proα2(I) mRNAs in the various tissues revealed a different pattern of mRNA sizes in the tissues which could synthesize type I procollagen and the chondrocytes, which could indicate that possible changes in the processing of the mRNAs had occurred, affecting the translatability.

Several factors have been shown to affect collagen synthesis in vitro. Leukotriene C_4 (LTC_4) was shown to stimulate collagen synthesis in rat lung fibroblasts (Phan et al, 1988). There appeared to be specific receptor sites for LTC_4 on fibroblasts. Phan et al (1988) point out that these observations could be important in wound healing after inflammation and injury, as activated phagocytes

would be likely to release leukotriene B_4 (LTB_4) which would chemoattract fibroblasts to the site, and LTC_4 which would result in increased collagen (and non-collagen protein) synthesis, thus promoting wound healing. Increasing the amount of collagen (i.e. procollagen) synthesized would possibly increase the action of the procollagen proteinases, resulting in more collagen being laid down.

Corticosteroids selectively decrease procollagen synthesis in fibroblasts and *in vitro*, associated with a decrease in total cellular type I procollagen mRNAs (for a review, see Cutroneo, Sterling and Shull, 1986). The synthetic glucocorticoid, dexamethasone, was shown to decrease the transcription of the proα1(I) and proα2(I) genes in 12 day chick embryo skin fibroblasts, and this was associated with increased binding specificity of three non-histone proteins for the proα2(I) promoter-containing fragment (Cockayne and Cutroneo, 1988). Dexamethasone treatment also reduced the synthesis of type I and type II procollagen in intact 17 day chick embryo tendons and sterna (Oikarinen *et al*, 1988), by reduction of the mRNA levels. Dexamethasone treatment had no effect on the conversion of procollagen to collagen as shown by pulse-chase techniques (Oikarinen *et al*, 1988). Specific glucocorticoid receptors were present in tendon fibroblasts and chondrocytes.

Transforming growth factor-β (TGFβ) was found to stimulate collagen synthesis in a dose-dependent manner in human dermal fibroblasts (Raghow *et al*, 1987), with corresponding effects on the levels of mRNAs. Assays of transcription revealed that the rate of transcription remained unaltered, so Raghow *et al* (1987) suggest that the effect of TGFβ is due to preferential stabilization of the procollagen (and fibronectin) mRNAs. TGFβ is known to promote a wound healing response *in vivo* which is characterized by increased cellular proliferation and protein synthesis. In foetal rat bone cells, TGFβ caused a

stimulation of protein synthesis, with a greater increase in type I procollagen synthesis (Wrana et al, 1988).

The monocyte derived tumour necrosis factor-α inhibited collagen production relative to other proteins in human fibroblasts, with a corresponding decrease in the level of the procollagen mRNA (Solis-Herruzo, Brenner and Chojkier, 1988). Interleukin-1α did not affect collagen synthesis in rabbit uterine cervical fibroblasts (Ito et al, 1988). Dihydroxyvitamin D_3 inhibits procollagen type I synthesis in the differentiated rat osteoblast (Rowe and Kream, 1982). The above papers are a selection and it should be borne in mind that effects may vary between tissues. The only study of processing was by Oikarinen et al (1988), who showed that dexamethasone treatment did not affect processing, although the synthesis of type I procollagen was decreased. As a decrease in the synthesis of type I procollagen would affect the enzyme:substrate ratio, it would seem that for the rate of processing to remain unaffected, the amounts of the processing enzymes would also have to change, in order for a constant enzyme: substrate ratio to be maintained. It would be interesting to see if there is co-ordinate regulation of the procollagen proteinases and procollagen synthesis, and if the levels of the mRNAs coding for the enzymes are affected, and also if the kinetics of processing are altered.

1.6 Fibril Formation

After secretion from the cell and proteolytic removal of the propeptides, collagens I, II and III assemble in the extracellular matrix as periodic fibrils (e.g. Prockop et al, 1979; Heathcote and Grant, 1980; Gross and Bruns, 1984; Kivirikko and Myllyla, 1982, 1984, 1985; Burgeson, 1988). The precise mechanism of fibril formation is unclear, however. Here the results of studies into the mechanism of fibril formation in vitro will be presented,

followed by a consideration of the possible *in vivo* mechanism. Factors which are thought to influence final fibril diameter will be considered.

1.6.1 Fibril structure

Collagen fibrils in the electron microscope when negatively or positively stained show a regular transverse banding pattern with an axial periodicity (D) of 60-65 nm. The alternating dark and light bands of the negative staining pattern show that each D-repeat can be divided into a "gap" zone penetrated by the stain and an "overlap" zone, which is relatively impervious to the stain (Figure 1.5). Positive staining reveals twelve staining bands, and this is thought to be due to the heavy metal ion reacting with the charged residues (for a fuller discussion of staining and the staining pattern, see Chapman, 1984; Chapman and Hulmes, 1984). In the presence of ATP at low pH, collagen molecules aggregate to form SLS crystallites with a length of 300 nm, corresponding to the length of the collagen molecule, indicating that the molecules have aggregated side by side. Superposition of positive staining patterns from SLS mutually staggered by integral multiples of D gives a staining pattern closely resembling that of a collagen fibril. Thus, molecules in a collagen fibril are staggered by n x D. The value of D has been found by comparing the fibril staining pattern with the pattern predicted from the sequence, using a range of values of D. The axial distribution of charge can be predicted from sequence data by summation of the five D-staggered molecular charge distributions (four in the gap zone). Agreement is best when D=234 residues (Chapman and Hulmes, 1984).

X-ray diffraction has been used to characterize the lateral packing and axial arrangement of molecules within the collagen fibrils. The axial D-repeating structure gives rise to the meridional reflections (i.e. parallel to

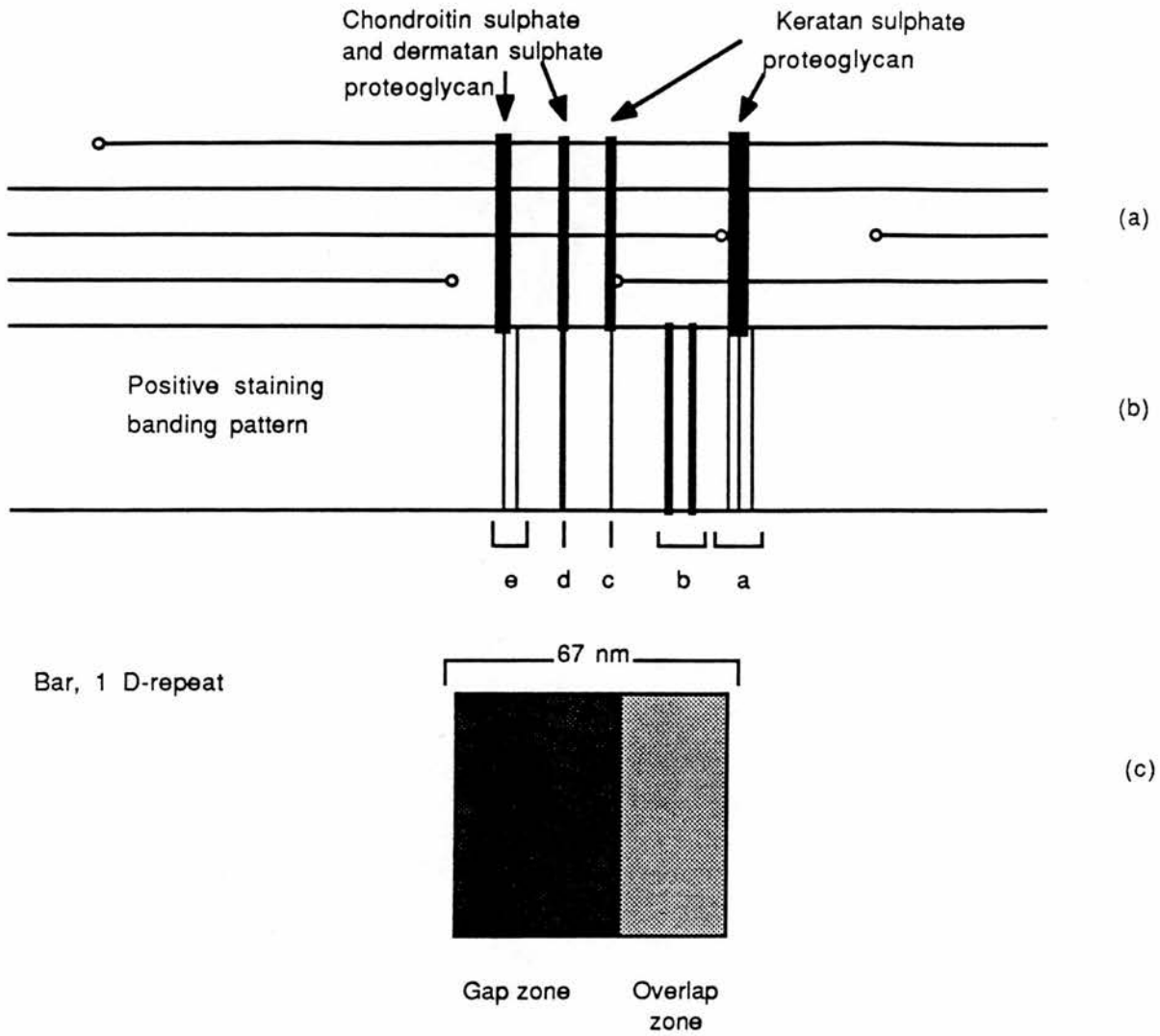

Figure 1.5 The a-e banding pattern within the D-period of the type I collagen fibril (b) is shown against the collagen molecules in a D-staggered array (a). The gap-overlap structure is also shown (c). The locations of chondroitin sulphate, dermatan sulphate and keratan sulphate proteoglycans are shown on the quarter-stagger diagram to correlate with the a, c, d, and e banding pattern. Bar, 67 nm (1 D-repeat). Adapted from Scott, 1988.

the fibre axis) in the diffraction pattern, and equatorial (perpendicular to the meridian) diffraction gives information about the lateral packing of the molecule (Cassidy et al, 1980). In an X-ray diffraction pattern of stretched rat tail tendon collagen, the near equatorial X-ray intensity is propeller shaped, and this indicates a tilting of the molecules with respect to the fibril axis.

The quasi-hexagonal packing model of Hulmes and Miller (1979) is the model which accounts for most of the X-ray data. The arrangement of collagen molecules in the quasi-hexagonal model is shown in Figure 1.6. It is convenient to visualize the fibril as a series of concentric circles, each circle being one molecule thick, and each circle being staggered by 1D relative to the next. A fibril appears in transverse section as a set of closely packed numbered circles, each representing a plane section through a molecular cylinder, the numbering showing the segment intersected. Thus, molecules on the outer surface of the fibril will be in non-staggered axial register, as are SLS, which may have significance for fibrillogenesis. It can be seen from Figure 1.6 that 4D-staggered molecules will be adjacent to one another, with the possibility of forming intermolecular crosslinks. In ultrathin cross-section, the fibrils seem to be composed of concentric circles or spirals with equally spaced tangentially oriented lines of density separated by 4.6 nm (Hulmes et al, 1981). Also, from this work, the fibril appears to be be composed of polygons of crystallinity, rather than circles. This was shown by Hulmes, Holmes and Cummings (1985) using image processing.

The electron microscopic results and the quasi-hexagonal model can be combined to give a model for fibril structure in which the molecules appear tilted by 4° when viewed from the side. Thus, the molecules appear to follow a helical path around the fibril, with one end of the molecule nearer the surface. The presence of the concentric layers is consistent with a model of fibril

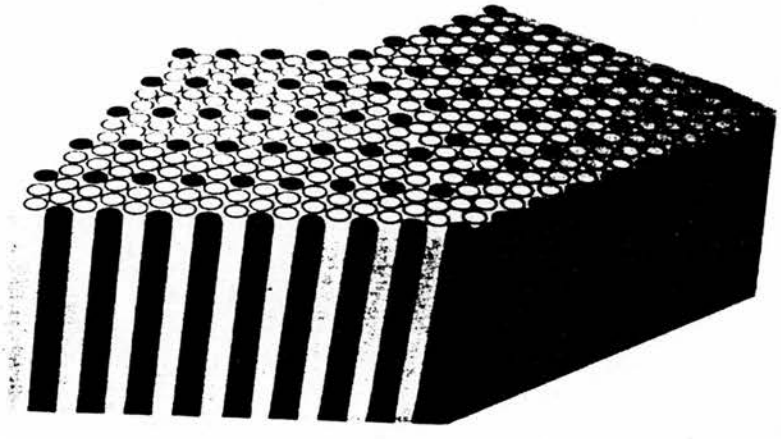

Figure 1.6 Perspective view of two adjacent crystalline domains in a segment of a model for the three-dimensional packing of molecules in a collagen fibril. Open circles portray sections through individual molecules and closed circles represent molecular ends, i.e. molecules indicated by open circles are staggered by nD (n=1,2,3 or 4) with respect to the molecules indicated by the closed circles. The two nearer surfaces are the exterior surfaces of the two domains. Molecules are tilted by about 5° in planes which make an angle of 30° with the exterior surface. (From Chapman and Hulmes, 1984, with permission).

growth by accretion of concentric layers of non-staggered collagen (Hulmes, 1983) and is consistent with the observation of Parry and Craig (1979) that fibril growth occurs in 8 nm increments.

In cornea, the microfibrils have an angle of about 18° to the fibril axis (Marchini et al, 1986). Examination of the axial distribution of the staining pattern of corneal collagen fibrils and tendon fibrils revealed extra regions of electron density in the gap zone and in the overlap zone which were attributed to sugar (Meek et al, 1981). Extra regions of density were seen by Marchini et al (1986). A glycoprotein of molecular weight 135,000 daltons has recently been proposed to account for this extra density (Wall et al, 1988).

In lamprey notochord the fibrils are not closely packed (Brodsky and Eikenberry, 1985). The fibrils are also less closely packed in developing tendon and in cornea than in adult rat tail tendon (Eikenberry et al, 1982; Brodsky and Eikenberry, 1985).

1.6.2 Fibrillogenesis In Vitro

It has been shown by numerous investigators that acidic solutions of collagen will spontaneously form aggregates when the pH and temperature are raised (Wood and Keech, 1960; Williams et al, 1978; Gelman, Williams and Piez, 1979). These aggregates have the characteristic D-periodicity seen in vivo. The fibril forming process can be followed spectrophotometrically by monitoring absorbance (related to the turbidity) at 313 nm or 400 nm (Figure 1.7). It shows a "lag" phase, a "growth" phase where there is a rapid increase in turbidity, and a plateau phase. The final absorbance can be correlated with the width of the fibrils (Wood and Keech, 1960). If fibril formation is initiated at 26°C for 10 minutes, cooled to 4°C for 40 minutes and then returned to 26°C, then the half time for fibril formation and the final turbidity

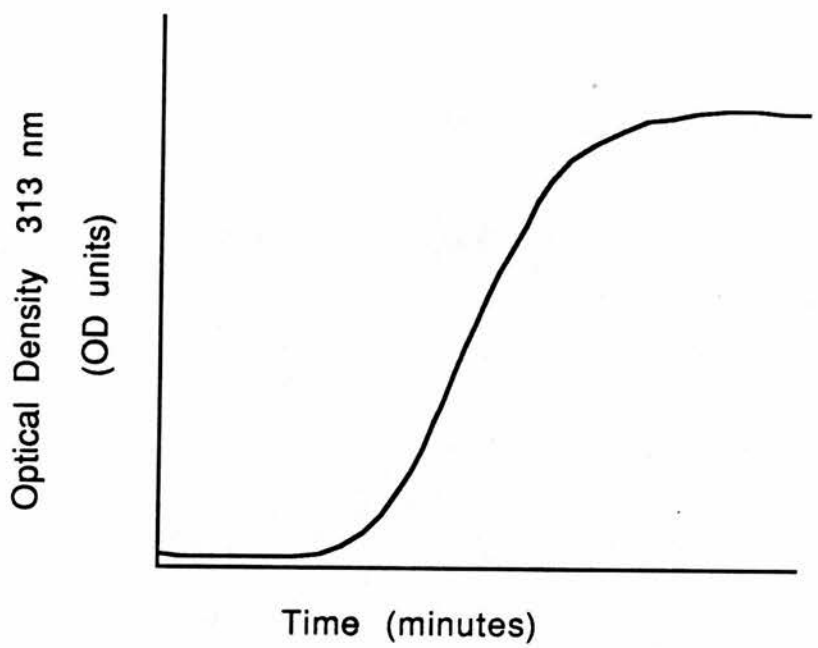

Figure 1.7 A typical turbidity curve showing the increase in optical density as collagen fibrils form from a solution of monomeric collagen molecules. As the pH and temperature are raised, there is a lag phase followed by a growth phase in which the turbidity increases.

were the same as in control samples. Thus, this suggests a three step process: 1) a temperature-dependent phase; 2) a temperature-independent phase without an increase in turbidity and 3) a temperature-dependent growth phase with an increase in turbidity (Gelman, Williams and Piez, 1979). The reaction times of steps 2 and 3 are inversely proportional to collagen concentration, which suggests that both linear and lateral growth occur by accretion. At the end of the lag phase, long thin filaments of diameter 2-4 nm were seen, although no periodic structure was visible. At the turbidity plateau, banded fibrils were visible. Na, Butz and Carroll (1986) presented evidence that fibril growth occurs by a process of nucleation and growth rather than accretion.

The initial aggregate formed is thought to consist of 2 collagen molecules staggered by 4D (Silver and Trelstad, 1979; Trelstad, Birk and Silver, 1982; Ward, Hulmes and Chapman, 1986). Rotary shadowing and visualization by electron microscopy revealed the presence of collagen monomers (240-280 nm in length) and a smaller number of filaments 400-580 nm in length, the amounts of which increased during the lag phase. The length of this aggregate is consistent with a dimer staggered by 4D. It is suggested that this is because telopeptide-helix interactions are greatest and contribute more to the total interaction energy when the overlap is least (Ward, Hulmes and Chapman, 1986). The 4D dimer grows in length by the addition of another monomer. These trimers then begin to associate laterally to produce wider intermediates (Trelstad, Birk and Silver, 1982). These intermediates can add to each other to form a larger subfibrillar structure. The morphology of the aggregates formed at various times was studied using electron microscopy (Trelstad, Hayashi and Gross, 1976). The first aggregates formed upon heating for up to 15 minutes had diameters of 5 nm, or multiples of 5 nm. These were laterally associated limiting initial aggregates as seen by negative staining. After 15 minutes

at 37°C, linear fibrillar aggregates appeared, which were similar in width to the initial aggregates. Initial aggregates were seen near the ends of linear fibrillar aggregates, and this suggests that growth occurs by addition to ends. Electron microscopy of the growth phase aggregates suggests that lateral growth occurs via the wrapping together of the subfibrils (Trelstad, Hayashi and Gross, 1976).

Several authors have shown that the telopeptides play important roles in fibril formation. For example, removal of the C-terminal telopeptide by carboxypeptidase treatment inhibited lateral assembly, while pepsin digestion inhibited linear assembly (Capaldi and Chapman, 1982). Complete removal of the telopeptides abolished fibril formation (Capaldi and Chapman, 1982).

Prockop and colleagues (Miyahara et al 1982, 1984; Kadler, Hojima and Prockop, 1987) have devised a fibril forming system which approximates more closely to the in vivo situation. Procollagen, or procollagen intermediates, are digested with the specific processing enzymes and the turbidity is monitored. The kinetics of this system and the collagen system are similar, except that the lag phase is longer. The increased length of the lag phase is accounted for by conversion of the intermediate to collagen. Using pC-collagen as a substrate and cleaving with C-proteinase enabled a value to be calculated for the critical concentration (Kadler, Hojima and Prockop, 1987). The value at 37°C is 0.42 μg/ml. Values of ΔG, ΔH and ΔS could be calculated. Assembly of collagen fibrils de novo is endothermic and entropy driven. The major driving force for fibril formation is the increase in entropy associated with the loss of water bound to the collagen monomer as polymerization occurs. These data support a nucleation-polymerization hypothesis.

As it is known that fibrils of type I collagen have differing ranges of diameters in different tissues in vivo (Section 1.6.3), it is of interest to examine fibril

formation of collagen extracted from different tissues *in vitro* to see if there are any differences in the rates of fibril formation. Corneal type I collagen forms fibrils 6-7 times more slowly than scleral type I collagen (Birk and Lande, 1981). The amount of lysine hydroxylation in cornea was 28.1% and that in sclera was 19.8%. Corneal hydroxylysine was four times more glycosylated than scleral hylys. The final absorbance for corneal and scleral collagen fibril formation was similar, indicating that the final diameter was not affected. Apparent first order rate constants for the lag and growth phases of fibril formation were smaller for corneal collagen than for scleral type I collagen and the activation energy of the growth phase for corneal collagen was greater than for scleral type I collagen (Birk and Silver, 1984b). Adult bovine corneal type I collagen had a longer lag phase, slower rate of growth and formed smaller fibrils than type I collagen extracted from adult and foetal bovine tendon and skin (Valli *et al*, 1986).

The influence of various purified ECM components on fibrillogenesis has been widely studied (Section 1.6.4). Trelstad, Birk and Silver (1982) suggest that other components mixed with collagens may create intermediates of fibril assembly with different or unique properties, and this may affect intermediate and final morphologies. Apart from the effects of PGs and other collagen types on fibrillogenesis, fibronectin was shown to accelerate the fibrillogenesis of type I collagen and delay the fibrillogenesis of type III collagen (Speranza, Valentini and Calligaro, 1987). Collagen types II, III, V and XI can also form fibrils *in vitro* (Lee and Piez, 1983; Adachi and Hayashi, 1985, 1986; Brodsky and Eikenberry, 1985; Broek *et al*, 1985).

1.6.3 Fibrillogenesis *In Vivo*

Several authors have suggested that procollagen SLS-

aggregates (Section 1.6.1) may be involved in the formation of D-staggered fibrils *in vivo* (e.g. Bruns *et al*, 1979; Weinstock and Leblond, 1974). SLS-like aggregates of pN-collagen were prepared by Veis *et al* (1973) and it was suggested that aggregates of pN-collagen may be incorporated into fibrils, and help register newly formed fibrils. "Parallel threads" were observed in cylindrical Golgi saccules of odontoblasts (Weinstock and Leblond, 1974) and in the predentin and dentin matrix of the rat incisor tooth (Weinstock, 1977). These structures corresponded to procollagen molecules arranged in an antipolar array with heads and tails in register. The "centrosymmetric" structures possessed tufts at either ends, which were interpreted as the N- and C-propeptides. Some centrosymmetric structures were seen in close association with collagen fibrils, so it was suggested that these structures were involved in fibril formation. SLS-like aggregates were also observed in vacuoles of tendon fibroblasts (Trelstad and Hayashi, 1979) and in secretory granules of fibroblasts of the rat foot pad (Marchi and Leblond, 1983). Autoradiography of the rat foot pad fibroblasts revealed that after 40-60 minutes of labelling, label appeared *over* collagen fibrils, and this was interpreted as collagen being added to existing fibrils, instead of forming new ones.

It has been suggested that collagen fibrils form in cytoplasmic recesses in fibroblasts, and that these recesses form compartments during morphogenesis. The recesses usually contained one fibril, although as many as three could be seen (Trelstad and Hayashi, 1979; Birk and Trelstad, 1984, 1985, 1986). Thus if fibrils were formed in these recesses, presumably processing is occurring here too. Dimeric SLS-like aggregates were seen, which is of interest since (e.g.) Ward, Hulmes and Chapman (1986) observed end to end dimeric aggregates as the first step in assembly *in vitro*. By autoradiography the recessed fibrils were shown to be labelled (Trelstad and Hayashi,

1979), but in contrast, no label or recesses were observed by Marchi and Leblond (1983), which could indicate that these fibrils were being endocytosed. The narrow recesses are proposed to fuse with each other to form bundles in a second "extracellular compartment", and a third compartment is proposed to consist of laterally associating bundles, surrounded by cell recesses and foldings of the cell surface (Birk and Trelstad, 1985). These recesses were also seen on corneal fibroblasts (Birk and Trelstad, 1984).

The deep recesses observed by Birk and Trelstad (1984, 1985, 1986) and the observations of SLS-like aggregates in vacuoles and recesses (Trelstad and Hayashi, 1979; Weinstock and Leblond, 1974; Marchi and Leblond, 1983) suggests that the cell surface influences aggregation and fibrillogenesis directly, either by directing the orientation of collagen fibrils and/or inducing aggregation. Bruns et al (1979) observed SLS-like structures on the substratum between human skin fibroblasts and in culture medium of 17-day chick embryo fibroblasts, and of other cultured cells, and in secretory vacuoles of fibroblasts and chondrocytes. It was proposed that procollagen is secreted in the form of SLS aggregates, and that processing of the procollagen SLS is accompanied by direct incorporation into the assembling fibril. Sedimentation coefficients indicated that only monomers were present, whereas electron microscopy showed aggregates (Hulmes, Bruns and Gross, 1983). Prolonged ultracentrifugation resulted in the procollagen monomers aggregating in the zero-D array, and thus it was suggested that the SLS aggregates form at high concentration which dissociate on dilution (Hulmes, Bruns and Gross, 1983). However, it was recently shown that these results and the results of Bruns et al (1979) were due to surface-induced aggregation, as procollagen at concentrations up to 800 µg/ml behaved in solution as a monomer, and was only aggregated when in contact with a surface during

preparation of samples for electron microscopy (Mould and Hulmes, 1987). However, these authors were unable to say whether or not aggregates might occur at the higher concentrations which probably occur *in vivo*.

The possible role of surface induced aggregation is of interest. Binding of type I collagen to the surface of fibroblasts has been observed in culture (Goldberg and Burgeson, 1982). This binding of type I collagen was competitively inhibited by types II and III procollagen, and by the N-propeptide of type I procollagen. The cyanogen bromide (CNBr) peptide α1CB8 also inhibited binding, indicating that ligands were distributed all over the molecule. It was proposed that this binding of monomers could induce aggregation and/or binding of aggregates to the cell surface, and this could be essential for fibrillogenesis. Immunocytochemical localization of type I procollagen in cultures of human fibroblasts revealed staining in a non-fibrillar, aperiodic form to the fibroblast surface (Phelps, Martinez-Hernandez and Goldberg, 1985). Several cell-surface collagen 'receptors' have recently been characterized (Dedhar, Ruoslahti and Pierschbacher, 1987; Pfäffle *et al*, 1988).

Data have been collated on collagen fibril diameters and distribution of diameters as a function of age (Parry, Barnes and Craig, 1978; Parry, Craig and Barnes, 1978; Parry and Craig, 1984). Collagen fibrils from foetal and newborn animals have a unimodal distribution of diameters. At maturity, the mass-average diameter of the collagen fibrils is generally larger than at birth and the distribution of fibril sizes may be either unimodal or bimodal, depending on the tissue. In all tissues examined except cartilage, the mean and mass-average diameters of the fibrils decrease beyond maturity. An attempt was made to correlate fibril diameter distribution with mechanical properties (Parry, Barnes and Craig, 1978). In altricious animals, the fibrils recommence growth after birth.

Precocious mammals (which are capable of locomotion immediately after birth) contain fibrils which are sufficiently developed at birth to cope with the mechanical demands made upon them. The tensile strengths and mechanical properties correlate with the mass-average diameter and collagen fibril diameter distribution respectively. Parry, Barnes and Craig (1978) postulate that large diameter fibrils will have a greater density of cross-links in order to provide greater tensile strength, and in small fibrils interactions between GAGs and collagen which may be important in inhibiting the occurrence of non-recoverable "creep" will be greatest. This would explain bimodal distributions; two populations of large and small diameter fibrils to endow a tissue with both tensile and creep-resistant properties. Oriented type I tissues which are subjected to long-term high stress have a bimodal distribution at maturity. The form of the fibril diameter at birth is sharp for altricious animals, but broad for precocious animals, i.e. crimp-resistant and tensile properties are found in the one population.

In altricious animals such as human and rat, diameters of skin fibrils increase from early foetal stages to maturity, after which time they decrease. In precocious animals such as sheep, skin fibril diameters are at a maximum at birth and then decrease. In both types of animal, however, the maximum diameter of the fibrils reached is 90 nm (Flint et al, 1984). In the skin of trout, which acts as a direct tendinous extension of the underlying muscles, the fibrils can grow up to 280 nm in diameter.

Collagen fibril diameters were investigated across the corneas of vertebrates (Craig and Parry, 1981). In contrast to some previous reports (Borcherding et al, 1975) fibril diameters remained constant across the cornea, except in chick, magpie and rat in areas adjacent to Descemet's membrane. In all species studied except bony fish and sea-lion, foetal and adult corneas had mean

diameters of 17 and 24 nm respectively. These values differ by approximately the 8 nm increment seen by Parry and Craig (1979). The diameters of fibrils in vertebrate corneas at birth have attained the diameters which are maintained throughout adult life. This implies that a very precise diameter regulating mechanism operates in cornea. This is necessary to maintain the light-scattering properties of the tissue which are required for transparency (Maurice, 1957; 1969).

1.6.4 Factors Involved in Fibril Diameter Regulation

In section 1.6.3 it was shown that in all tissues examined except for cornea, the mass-average diameter changes between birth and maturity, and between maturity and senescence. This implies that factors which influence fibril diameter might be important during development. Several factors have been proposed to be important in diameter regulation of fibrils, and these will be considered below.

1.6.4.1 Proteoglycans and Glycosaminoglycans

A correlation was found between increasing collagen fibril diameters and decreasing GAG content across artery walls, going from the outer adventitia layer to the inner intima layer (Merrilees, Tiang and Scott, 1987). A high concentration of chondriotin sulphate (CS) was found to correlate with low mean fibril diameter. These data do not fit the suggestion of Parry, Barnes and Craig (1978) that wide fibrils are associated with tensile stress, as the greatest stress in artery walls is highest at the lumen, where the fibrils are smallest. In skin, hyaluronic acid (HA) was found to be chiefly associated with immature tissues during development, and dermatan sulphate (DS) is associated with more mature tissues with increased diameters (Flint et al, 1984). Collagen fibril

organization and PG content were also examined on the transition zone from cornea to sclera. The major GAG present in the central cornea is keratan sulphate (KS). In the limbus region between the cornea and sclera, the mean fibril diameter and the range of diameters greatly increases, with a resulting decrease in the number of fibrils per unit area, and a major decrease in the concentration of GAGs, mainly due to a loss of KS. DS becomes the predominant GAG (Borcherding *et al*, 1975).

In vitro effects of PGs on fibrillogenesis have also been noted. Chondroitin sulphate appears to promote fibril formation (Hayashi and Nagai, 1972), although this contrasts with the finding that chondroitin sulphate GAG chains (CS-GAG) had little effect on the fibril-forming process (Snowden and Swann, 1980). Dermatan sulphate GAG chains (DS-GAG) and HA (HA at a concentration of greater than 100 μg/ml) decreased the lag time, increased the rate of fibril formation and slightly increased the final absorbance. A high density PG from articular cartilage containing CS prolonged the lag time, but had little effect on the rate of growth (Snowden and Swann, 1980). The small dermatan sulphate proteoglycan (DS-PG) of tendon inhibited the fibrillogenesis of types I and II of collagen *in vitro*. None of the large PGs inhibited this process (Vogel, Paulsson and Heinegård, 1984). Alkali treatment of the small DS-PG of tendon abolished its ability to inhibit fibrillogenesis, whereas chondroitinase treatment did not, indicating that the interaction depends on the core protein, rather than the GAG chains. The presence of the small DS-PG of tendon was found to decrease the rate of fibril formation and the final fibril diameter (Vogel and Trotter, 1987), suggesting that it inhibited the lateral aggregation of the collagen fibrils being formed. Human uterine cervical small DS-PG did not affect the rate of fibril formation, but increased the final absorbance by up to 90%. Core protein or the side chains alone did not have this effect. The fibril

diameter was not affected, but in the presence of the PG laterally aligned bundles of fibrils were obtained (Uldbjerg and Danielsen, 1988). They suggest that there are two populations of DS-PGs, one which decreases the rate of fibril formation and one which increases the final absorbance. Link protein, which in cartilage binds to the core protein and HA, stabilizing the interaction between these two molecules, was found to have no effect on the kinetics of fibril formation or the size of the fibrils. Link protein together with cartilage PG or bone PG increased the rate of aggregation and the fibril diameter (Chandrasekhar et al, 1984).

Vidal (1986) showed that chondroitin 4- and 6- sulphates increased optical retardation due to birefringence because of an oriented binding to the collagen fibrils. Borcherding et al (1975) suggest that the repulsion of the negatively charged GAG chains would orient the protein core perpendicular to the long axis of the fibril, and this would create a negatively charged field around the fibre, which would maintain the spatial relationships between the individual fibres.

There is a slight negative correlation between the amount of HA (and to a lesser extent CS) and the amount of collagen, which suggests that as the amount of collagen/g tissue increases, there is less space for HA or PG and their total proportions then decline, i.e. HA and CS are in the interfibrillar space (Scott and Hughes, 1986). DS is thought to be on the periphery of the fibril, as a plot of the ratio of hypro:GAG against the fibril radius is a straight line. Scott, Orford and Hughes (1981) showed that the change in population from thin, uniform fibrils to one of varying larger diameters coincided with a decrease in CS and HA concentration in rat tail, chick and calf tendon. Use of the dye cupromeronic blue permits ultrastructural localization of PGs and counterstaining of the fibril band pattern allows the identification to be made with the collagen staining bands (Scott and Orford,

1981; Chapman and Hulmes, 1984; Scott and Haigh, 1985). In this way, the small DS-PG of tendon has been shown to bind at the d/e band of the positive staining pattern, corresponding to the gap zone (Scott and Orford, 1981), where cross-linking occurs, so the authors suggest that association of DS-PG here will restrict lateral growth (Figure 1.5). In pig aorta small fibrils were associated with high concentrations of DS. From the inner areas of the wall to the outer areas fibril diameters increased, correlating with a fall in the amount of DS-PG (Merrilees, Tiang and Scott, 1987). Keratanase-sensitive and chondroitinase-insensitive PG filaments of rabbit cornea are located at the a and c bands of the positive staining pattern, representing the "step" of the gap/overlap zone (Figure 1.5) (Scott and Haigh, 1985).

Mixing of type XI collagen with heparin, CS-GAG or CS-rich PG inhibited fibril formation and resulted in the formation of aggregates with electron dense cores and shortened, branched fibres (Smith, Williams and Brandt, 1987). However, the pepsin-extracted form of type XI used in these studies is not the form of this collagen species present in the tissue (Brodsky and Eikenberry, 1985; Eyre and Wu, 1987).

1.6.4.2 Amount of Hydroxylysylglycosides

It has been suggested that the amount of glycosylation of the collagen molecule affects the final fibril diameter (e.g. Grant et al, 1969). It was found that a high carbohydrate content correlated with a low mean fibril diameter, with only a narrow range of diameters, as in cornea, where 73% of the hylys is glycosylated (Schofield, Freeman and Jackson, 1971). In tendon, only 6% of the hylys is glycosylated and there is a wide range of diameters with a large mass-average diameter. During development of rabbit Achilles tendon, the amount of hylys glycosides decreases as the fibril diameters in the tendon

increase (Cetta et al, 1982). A rapid decrease of the di:monosaccharide ratio was observed during foetal life and there was a much lesser decrease during postnatal life. An enzyme activity which could possibly remove the disaccharide from the collagen leaving a monosaccharide, accounting for the decrease in the di:mono ratio was observed by De Luca et al (1983).

The presence of sucrose and glucose inhibited fibril formation in an in vitro system (Hayashi and Nagai, 1972). Sucrose at concentrations of 0.1 M and 0.15 M decreased the final opacity (related to the fibril width). Fibril formation was studied with calf skin collagen which was then enzymatically glycosylated or deglycosylated (Amudeswari, Liang and Chakrabarti, 1987). Deglycosylated collagens had a shorter lag time and a faster growth rate than glycosylated collagens. The degree of decreased fibril formation correlated with the loss of ability of collagen to act as a substrate for lysyl oxidase in the presence of glucose (Lien et al, 1984).

It has been proposed that increased glycosylation lowers the surface energy of collagen fibrils by increasing the hydrogen bonding, thus hindering collagen-collagen interactions and making large diameter fibrils less likely (De Luca et al, 1983; Birk and Silver, 1984b). The presence of sugar residues attached to the hylys side chain may result in an increase in length of the side chain, which may have to be redirected axially before lateral growth can occur, delaying packing of the collagen molecules and inhibiting growth of the fibrils (Birk and Silver, 1984b).

Localization of the sugar residues in the CNBr fragment α1CB3 showed that most of the sugar is attached to a lys residue which must occur in the overlap zone (Panjwani and Harding, 1978). The presence of sugar residues in the overlap zone might impede accretion of the collagen molecules (Harding, Crabbe and Panjwani, 1980). At least some of the glycoside residues identified in the CNBr

fragment α1CB6 must be in the overlap region, because only one of the lys residues in the Y position of the sequence is in the gap zone (Harding, Crabbe and Panjwani, 1980). A decrease in the electron density difference between the gap/overlap zone could possibly be due to the presence of hylys glycosides (Meek et al, 1981; Marchini et al, 1986). However, this has been attributed to the presence of the non-collagenous portion of type V collagen which could be present in corneal collagen fibrils (Adachi and Hayashi, 1987), and to a glycoprotein of molecular weight 135,000 daltons (Wall et al, 1988). Sugars could influence the rate of conformational changes in the telopeptides which are necessary for fibril formation (Helseth and Veis, 1981; Capaldi and Chapman, 1982).

The amount of hylys glycosylation will be dependent on the rate of triple helix folding, or on the ratio of the glycosylating enzymes to substrate. As the rate of triple helix formation is the same in tendon and cornea (Kao et al, 1983), the differing amounts of hylys glycosylation could be due to differences in the enzyme-substrate ratio.

1.6.4.3 Interactions Between Collagen Types

In comparative studies of in vitro self-assembly, collagen type III formed fibrils more quickly than type II which generally formed fibrils quicker than type I (Birk and Silver, 1984a). The final turbidity was greatest for type I, then type III and then type II. The rates of the lag and growth phases were greatest for type III and slowest for type I, with intermediate rates for type II. However, these collagens were extracted with pepsin, which may have removed the telopeptides. As the presence of the telopeptides is necessary for fibril formation (Capaldi and Chapman, 1982), the relevance of these studies to other in vitro and in vivo work is not clear.

The fact that in vitro formation of collagen fibrils of

types I, II, and III produces fibrils of different diameter (Birk and Silver, 1984a) and that tissues do not consist exclusively of one collagen type (Table 1.1), has led to the proposal that different collagen types may be present in a single fibril, and that the proportion of the two types in a fibril results in different fibril diameters. Collagen types I, II, III, V and XI can all form fibrils (Table 1.1; Miller, 1985). Lamprey notochord contains enough type XI collagen (5-10%) to be present on the surface of type II fibrils, and a surface location of type XI is supported by antibody studies of type XI in the vitreous humour (Brodsky and Eikenberry, 1985). Type XI collagen contains an extra globular N-terminal domain which may limit the diameters of type II fibrils by steric effects (Brodsky and Eikenberry, 1985) as proposed by Hulmes (1983) and Chapman (in press) (Section 1.6.4.5). Type XI collagen can be reconstituted into fibrils *in vitro*, and these have diameters of 20 nm (Brodsky and Eikenberry, 1985) and 20-30 nm (Smith, Williams and Brandt, 1987).

In vitro, type V can be reconstituted into fibrils of 22 nm diameter with no axial periodicity (Brodsky and Eikenberry, 1985; Broek *et al*, 1985). Mixtures consisting of varying proportions of pepsin-extracted type I and V collagen resulted in fibrils of diameters which vary depending on the relative amounts of each type. Type V alone resulted in fibrils of diameter 38.2 nm, and a 1:1 mixture of type I and V produced fibrils of diameter 46.1 nm. The diameter distribution was sharp and unimodal, indicating that only one population of fibrils was formed (Adachi and Hayashi, 1986). Increasing the amount of type I collagen resulted in fibrils with larger diameters; 106.5 nm for a 3:7 mixture of type V:I and 142.9 nm for type I alone. Examination of the negative staining pattern showed that the difference in electron density between the gap/overlap zone was very small when more than 50% type V was present (Adachi and Hayashi, 1987). These authors

suggest that this is due to the presence of the globular N-terminal domain of the type V molecule in the gap zone.

Significant amounts of type V collagen have been reported in the chick corneal stroma, with values quoted ranging from 5% (Kao, Mai and Chou, 1982) to 20% (Pöschl and von der Mark, 1980). Using immunofluorescence, type V can first be detected in the corneal stroma of the embryonic chick from day 5-6 onwards, and is present throughout the stroma at day 9 (Linsenmayer, Fitch and Mayne, 1984). Treatments which disrupt the fibrillar structure result in greater accessibility of the type V epitopes to anti-type V antibody (Fitch et al, 1984; Linsenmayer, Fitch and Mayne, 1984; Linsenmayer et al, 1985). This masking of type V by type I occurs at all stages of development, which suggests that the type V is masked by type I soon after synthesis. Digestion with vertebrate collagenase correlates with the amount of unmasking, which implicates type I as the masking agent. Thus, the collagenase results and the existence of only a single population of fibrils in the cornea (Craig and Parry, 1981) suggests that type I and type V co-exist in the same fibril. Immunoelectron microscopy shows that corneal fibrils label with antibodies against type I and type V (Linsenmayer et al, 1985; Birk, Fitch and Linsenmayer, 1986). Recent work (Birk et al, 1988) using antibodies conjugated to different sized immunogold particles has shown that type I and type V are present in the same fibril.

Lapière, Nusgens and Piérard (1977) reconstituted fibrils of types I, III and mixtures of types I and III in vitro. The lag phase for the mixture was intermediate in length between the lag phases for type I alone and type III alone. A single population of fibrils was formed from the mixture. The values of the diameters of the mixture appear to be intermediate between those of the type I fibrils and those of the type III fibrils. Cross-links have been isolated from a human leiomyoma, which appeared

to cross-link the telopeptides of types I and III collagen, indicating that these two types coexisted in the same fibril (Henkel and Glanville, 1982). Recent work has shown that type III collagen is present on the surface of all fibrils in tendon skin, amnion and aorta, arguing against a role of type III in limiting fibril diameter (Keene et al, 1987).

The presence of types I and V in the same fibril leads to the question of how the non-collagenous portion of type V collagen could regulate type I diameter as proposed by Hulmes (1983) and Chapman (personal communication), as it appears to be buried or present as a core inside the type I, instead of being on the surface of the fibril (Linsenmayer, Fitch and Mayne, 1984; Linsenmayer et al, 1985; Birk et al, 1988; Fitch et al, 1988). It is also unclear how the presence of type III could limit the diameter of type I containing fibrils. Type V procollagen is secreted 100 minutes after synthesis begins (Fessler and Fessler, 1987) whereas type I is secreted 25 minutes after synthesis in cornea (Kao, Mai and Chou, 1982). The processing time of type I procollagen is much less than that of type V procollagen, where processing appears to take several hours. These facts pose questions as to the mechanism of masking of type V by type I. It is not known whether or not type V is processed by the same enzymes as type I collagen. The existence of separate N-proteinases for types I and III collagen and possibly for type V collagen suggests another level of control for the incorporation of two different types of collagen into fibrils.

Type IX collagen has been located on the surface of type II fibrils by rotary shadowing with a spacing of one D-period (Vaughan et al, 1988) and the N-terminal region of the COL2 domain contains a hylys residue involved in cross-linking to α1(II) at the N-telopeptide (van der Rest and Mayne, 1988). Assuming that type II and type IX are oriented in the same direction, this would place the GAG

chain in the gap zone of the fibril. This arrangement would place the COL3 arm out of the fibril, and this is supported by the rotary shadowing data of Vaughan et al (1988). Pepsin digestion revealed an apparent stoichiometry of type II:IX:XI of 8:1:1 on SDS-PAGE of chick sternal cartilage fibrils (Vaughan et al, 1988).

1.6.4.4 Procollagen Processing

In order for fibrils of the correct cross-section and diameter to be formed, propeptide removal must occur.

Using an in vitro system with the purified procollagen and the purified N- and C-proteinases at 37°C, Miyahara et al (1982, 1984) showed that varying the enzyme concentration had no effect on fibril diameter. pC-collagen would not form aggregates without removal of the C-propeptide (Miyahara et al, 1984). Thus, it was concluded that the differences in diameter were not explained by differential rates of cleavage, but rather by which intermediate is the immediate precursor of collagen, pC-collagen or pN-collagen. Thus, the order of cleavage may control the fibril diameter and the rates of cleavage may not be involved. However, in a system where both enzymes are present, variation of the rate of cleavage at either end could influence the final diameter. A greater amount of C-proteinase will give pN-collagen as the observed intermediate and this might lead to narrower fibrils. A greater amount of N-proteinase will give a pC-collagen intermediate and this might lead to wider fibrils.

Fleischmajer and colleagues (1981, 1983, 1985, 1986, 1987a, 1987b) have also investigated the role of procollagen processing in collagen fibril formation. Type I N-propeptide seems to be limited to small diameter fibrils and disappears from the surface of the fibril as growth occurs. The type III N-propeptide persists at maturity in the chick. A similar situation is seen in

foetal and adult human skin (Fleischmajer, Perlish and Timpl, 1985). The C-propeptide of type I procollagen seems to be associated with fibrils of more than 40 nm in diameter in 21-day embryonic chick skin (Fleischmajer, Perlish and Olsen, 1987a) and embryonic chick tibia (Fleischmajer, Perlish and Olsen, 1987b). Thus, Fleischmajer and colleagues propose that in small fibrils less than 40 nm diameter, pN-collagen is incorporated into fibrils, and this limits growth. After this, the N-propeptide is removed and further growth to mature diameters can occur. In type III fibrils, the N-propeptide is retained when the fibril reaches 40-65 nm. Processing stops and thus growth ceases. The further lateral growth of type I fibrils must occur via other mechanisms, possibly by the C-propeptide (Fleischmajer, 1986). Therefore, Fleischmajer suggests that the differential staining of large and small fibrils with anti-C-propeptide and anti-N-propeptide antibodies may be a reflection of differential rates of processing at the N- and C- ends of the procollagen molecules at the surface of large or small fibrils. This picture is complicated by the work of Keene et al (1987), who showed that all 67 nm periodically banded fibrils in human dermis uniformly labelled with monoclonal antibodies against type III collagen, regardless of fibril diameter, tissue age or location within the dermis, in contrast to the data of Fleischmajer. Hence, the significance of the data of Fleischmajer is unclear.

Antibodies directed against the C-propeptide of type II procollagen detected a 60 nm periodic labelling along type II fibrils in 2 day chondrocyte culture (Ruggiero et al, 1988). The fibrils were 15 nm in diameter. At 3 days of culture, the fibrils were still 15 nm in diameter, but most of the C-propeptide had disappeared from the fibrils. Thus, this argues against a role of the C-propeptide in the regulation of fibril growth, and Ruggiero et al (1988) suggest that the C-propeptide has a role in the initial

steps of fibril formation. In these experiments it was not known whether the C-propeptide was present in the form of pC-collagen, or if the C-propeptide had been enzymatically removed by the action of C-proteinase and was merely associated with the fibrils.

It could be suggested, then, that processing via pN-collagen leads to small fibrils, while processing via pC-collagen leads to wide fibrils. The diameters of fibrils in different tissues containing the same collagen type can differ greatly, as in tendon and cornea (e.g. Grant et al, 1969; Schofield, Freeman and Jackson, 1971; Parry and Craig, 1984), so it seems that the route of processing in cornea might be different from tendon. The situation with type III is difficult to interpret, as the results of Keene et al (1987) indicate that type III always forms heteropolymeric fibrils with type I collagen, except in cornea. Thus, the route of processing of type III might change with fibril diameter.

1.6.4.5 Models for the Regulation of Collagen Fibril Diameter

Possible factors involved in the regulation of fibril diameter have been discussed (Section 1.6.4). The actual mechanism of diameter regulation is unknown, however. A model has been proposed by Hulmes (1983) which takes into account quasi-hexagonal packing. As it has been observed that small fibrils seen to have the N-propeptide associated with them (Fleischmajer et al, 1981, 1983, 1985, 1987a,b), Hulmes suggests that a surface layer of pN-collagen molecules sterically prevents any further increase in fibril diameter. Temporary activation of the N-proteinase could result in removal of the N-propeptide and addition of a further layer of pN molecules onto the fibril surface. If N-proteinase acts preferentially on an aggregated substrate, then pN-collagen molecules in the interfibrillar space may not be significantly processed

during the short burst of N-proteinase activity. The radius will increase by 3.8/5 = 0.76 nm for every sheet of molecules added, and growth will occur until inhibition by steric blocking occurs (when 5 layers of pN-collagen have been added). As the width of five layers is 3.8 nm, the fibril diameter at all locations will increase by 2 x 3.8 nm (8 nm).

However, the Hulmes model is unsatisfactory in several respects. It does not account for the growth of wide fibrils which Fleischmajer showed were not labelled with antibodies against the N-propeptide, so regulation of diameter by the N-propeptide cannot operate on wide fibrils. Also, the Hulmes model would require precise "on -off" switching of the procollagen N-proteinase. Fleischmajer et al (1981) did show that it was possible for the N-propeptide to be removed from the surface of fibrils by the action of N-proteinase. The Hulmes model is also unsatisfactory because it assumes that the 4 nm spacing in the quasi-hexagonal lattice is equivalent to the 4 nm increments in radius observed by Parry and Craig (1979). This cannot be the case, as the 4 nm spacing of the quasi-hexagonal lattice was obtained from X-ray diffraction of hydrated samples, whereas the observations of Parry and Craig were of specimens dehydrated for electron microscopy. As Chapman points out, (in press), the true increment of Parry and Craig taking dehydration into account may be 5-6 nm and no such values of spacings occur in the quasi-hexagonal lattice. Also, many tissues do not show quasi-hexagonal packing, with only diffuse equatorial reflections. Thus Chapman (in press) has devised another model of regulation. The model does not require quasi-hexagonal packing, although near close packing is assumed. In the Chapman model, lateral growth is inhibited due to the presence of a "growth inhibitor" which could be a propeptide, a PG or any molecule associated with a specific region of the collagen molecule. If each molecule is divided into sections

labelled 1, 2, 3, 4 and 5 (corresponding to 1D, 2D, 3D, 4D and 4.4D) then in any cross section n/5 molecules will be labelled 1, 2 etc. If some pN-collagen molecules are present, then sections intersecting N-propeptide domains in a fibril will contain n/5 N-propeptides. If the N-propeptide is confined to the surface of the fibril, then when all n/5 N-propeptides are in contact forming a continuous layer around the fibril, no further growth can occur. This model can be reconciled with the experimentally observed diameters (Parry and Craig, 1979).

1.7 Defects in Processing and Fibrillogenesis

Defects have been observed on fibrillogenesis and processing in several genetic diseases. The best characterized of these defects is dermatosparaxis, a disease of sheep and cattle, in which the N-proteinase is deficient (Lapière, Lenaers and Kohn, 1971; Lenaers *et al*, 1971; Shoshan *et al*, 1974). A similar defect has been observed in cats (Patterson and Minor, 1977). Deficiency of the N-proteinase results in the formation of fibrils from pN-collagen which are thin, poorly organized, and irregular in cross-section (Lapière and Nusgens, 1974; Piérard *et al*, 1986). The defective form of the fibrils has been reproduced *in vitro* (Hulmes *et al*, submitted for publication). Collagen extracted from dermatosparactic tissues forms fibrils with smaller diameters as seen in the electron microscope (Lapière and Nusgens, 1974; Miyahara *et al*, 1983). The major tissue affected by this defect is skin. The N-propeptide of the type III collagen has been shown to contain greater amounts of complex mannose oligosaccharides than control type III N-propeptides although the relevance of this to the disease process is not clear (Shinkai and Lapière, 1981). A 34,000 dalton membrane protein which is related to the collagen binding protein anchorin CII is absent in dermatosparactic skin fibroblasts (Mauch *et al*, 1988). This may be involved

in the presentation of procollagen to the N-proteinase, so accumulation of pN-collagen in dermatosparactic skin could result from a combination of the defects in the N-proteinase activity and the binding protein.

The equivalent disease in man to dermatosparaxis is Ehlers-Danlos syndrome VII (EDS VII) (Holbrook and Byers, 1982). Holbrook and Byers (1982) obtained skin biopsy samples from patients with inherited disorders of connective tissue metabolism, in order to try to classify the defect in fibrillogenesis with the defect at the biochemical level. It was found that in many cases, a heterogeneous defect at the molecular level leads to a clearly identifiable syndrome with heterogeneous defects in the collagen and elastic fibrils. Defects in procollagen processing have been identified in cases of osteogenesis imperfecta (De Wet et al, 1983; Sippola, Kaffe and Prockop, 1984; Minor et al, 1986) and Ehlers-Danlos syndrome (Lichtenstein et al, 1973; Halila, Steinmann and Peltonen, 1986; Minor et al, 1986), due to defects in the levels of procollagen N-proteinase (Lichtenstein et al, 1973; Halila, Steinmann, and Peltonen, 1986) or from structural mutations which render pN-collagen resistant to cleavage (De Wet et al, 1983; Sippola, Kaffe and Prockop, 1984; Minor et al, 1986). Thus, mutations affecting the processing of type I procollagen can lead to more than one clinical syndrome. It seems that heterogeneous defects at the molecular level can lead to diseases with the same pathology.

Alterations in the amount of collagen can occur in acquired diseases, such as arthritis and emphysema (for references, see Laurent, 1987) and fibrosis (Laurent, 1985).

1.8 Introduction to the Present Study

In this chapter, it has been shown that both the kinetics of collagen fibril formation and the final

morphology of collagen fibrils may depend on several factors: 1) the extent of post-translational glycosylation of the collagen (and hence the activity of the glycosylating enzymes); 2) the rate and route of conversion of procollagen to collagen; 3) the presence or otherwise of genetically distinct collagen types in the same fibril; 4) the presence of extracellular matrix components such as PGs, GAGs, fibronectin etc; 5) any factors which affect 1)-4) above, such as hormones, growth factors and cytokines. Evidence in favour of an involvement of each of the above factors 1) - 4) in the regulation of fibril diameter has been presented.

The present study was undertaken to investigate the possible involvement of procollagen processing in fibril diameter regulation (Section 1.6.4.4; Fleischmajer, 1981, 1983, 1985, 1987a,b). Previous studies of procollagen processing have merely examined the apparent route of procollagen processing on SDS-PAGE after carrying out pulse-chase experiments (Davidson, McEneany and Bornstein, 1975; Fessler, Morris and Fessler, 1975; Morris et al, 1975; Uitto and Lichtenstein, 1976; Fessler and Fessler, 1979; Leung et al, 1979; Limeback and Sodek, 1979; Sodek and Limeback, 1979; Fessler, Timpl and Fessler, 1981; Sonohara et al, 1981; Gerstenfeld et al, 1984; Bateman et al, 1986; Minor et al, 1986; Gerstenfeld et al, 1988). This type of experiment gives no information about the rate constants of the individual steps of processing: procollagen to pC-collagen, procollagen to pN-collagen, pC-collagen to collagen or pN to collagen (Figure 1.4). As yet the rate constants for these reactions have not been determined. Hence, it was important to determine the rate constants of these steps by regression analysis of pulse-chase data to see whether preferred routes of processing really do operate, or if all four reactions proceed simultaneously.

If procollagen processing rate constants are influencing the final fibril diameter, then it might be expected that

as diameters change during development there would be corresponding changes in the rate constants. Fibril diameters in tendon increase between birth and maturity and then decrease between maturity and senescence (Parry, Barnes and Craig, 1978; Parry, Craig and Barnes, 1978; Parry and Craig, 1984). Fibril diameters of embryonic chick tendon also increase (Eikenberry, Brodsky and Parry, 1982; Eikenberry et al, 1982). Hence, if the rate constants for the processing of procollagen change during development, then this might be a further indication that intermediates of procollagen processing are involved in the regulation of fibril diameter.

Initial studies on the effect of tissue specific differences in type I collagen glycosylation on fibril formation are also reported. Collagen fibrils were generated in vitro by incubation of corneal and tendon type I procollagen at 37°C with purified procollagen N- and C-proteinases. The morphology of the collagen fibrils produced by this method was examined to see if there were any differences in the fibril forming process for the two samples which could be related to differences in the extent of collagen glycosylation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Purification of Tendon Procollagen

2.1.1 Materials

Fertilised hen eggs were supplied by Ross Breeders, Ltd (Newbridge, Midlothian). L-5-^{3}H proline (30 Ci/mmol; 1.11 TBq/mmol) was obtained from Amersham. Bacterial collagenase (isolated from Clostridium histolyticum) was supplied by Boehringer. Minimal Essential Medium (MEM) containing Earle's Salts and L-glutamine, Hank's Balanced Salt Solution (HBSS), trypsin (2.5%), foetal calf serum (FCS) and penicillin-streptomycin (PS, 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin) were all obtained from GIBCO. Scintran Cocktail T was supplied by BDH. Pepsin, N-ethylmaleimide (NEM) and phenylmethylsulphonylfluoride (PMSF) and BioGel P6DG were purchased from Sigma. N-hydroxysuccinimidobiotin was purchased from Pierce. ^{125}I-labelled streptavidin (4.5 x 10^{7} dps/mg) was a gift from Dr D K Apps. Sodium cacodylate and paraformaldehyde were obtained from Agar Aids. All other chemicals were supplied by BDH or Sigma.

2.1.2 Preparation and Incubation of Cells

The method used was a modification of that of Dehm and Prockop (1971), and Prockop and Tuderman (1982). Semi-sterile procedures were used throughout. Dissection instruments, Erlenmeyer flasks (Nalgene) and Swinnex filters (Millipore) were autoclaved prior to use.

The leg tendons from 30 dozen 17 day old chick embryos were dissected out in Hanks Balanced Salt Solution (HBSS), containing 1% PS. After isolation of the tendons, feathers and traces of blood were removed by washing three times in HBSS. The wet weight of the tendons was determined. A typical yield from 30 dozen chick embryos was 12 g. The tendons were digested at 37°C in an Erlenmeyer flask in

MEM (3 ml/g tissue) containing 2.5% trypsin (0.33 ml/g tissue) and collagenase (22 mg/g tissue) under 5% CO_2/95% air. The tendons were incubated on an orbital shaker (Lab Therm Lab Shaker, Adolf Kühner, Basel) or in a shaking water bath (Grant) at 37°C for about 1 hour, until only a few fragments of undigested tendon remained. Care was taken to avoid overdigestion with the trypsin and collagenase, as this could lead to cell rupture.

After the digestion was complete the digest was filtered through two layers of lens paper in a Swinnex filter. The flask was rinsed with modified Krebs II medium (111.2 mM NaCl/5.4 mM KCl/1.3 mM KH_2PO_4/1.3 mM $MgCl_2$/4 mM $NaHCO_3$/ 12.5 mM $NaHPO_4$/3.1 mM NaH_2PO_4/13 mM glucose, pH 7.3) containing 10% foetal calf serum (FCS) and 1% PS, and the washings were then filtered. The FCS contains inhibitors which prevent further action of the collagenase and trypsin.

The filtrate was added to the cell suspension and more Krebs II was added until the total volume was twice that of the digestion mixture. The suspension was centrifuged for 6 minutes at 1800 rpm (450 g) in a bench top centrifuge (MSE). The supernatant was removed and the pellet was resuspended by gentle pipetting in 30 ml Krebs II containing 10% FCS, using a sterile 10 ml pipette. Careful resuspension was essential since Dehm and Prockop (1971) found that using a vortex mixer damaged the cells. The cell washing was repeated twice with Krebs II/10% FCS and the pellet obtained from the final centrifugation was then resuspended in 20 ml of serum-free Krebs II (with 1% PS). The cell yield was determined using a haemocytometer (Weber). A typical yield from 30 dozen chick embryos was 6 x 10^9 cells. The cells were then diluted in an Erlenmeyer flask with Krebs II/1% PS to give a final concentration of 1 x 10^7 cells/ml. Ascorbate was added from a freshly prepared stock solution of 2 mg/ml to give a final concentration of 50 μg/ml. Radiolabelled proline was added to a final concentration of 1 μCi/ml (37 kBq/ml). The

cells in the flask were gassed with 5% CO_2/95% O_2 and then incubated on the orbital shaker at 37°C for 5 hours.

2.1.3 Medium Collection and Polyethylene Glycol Precipitation

After incubation on the orbital shaker, the medium was centrifuged for 3 minutes at 1800 rpm (450 g). The supernatant was collected and centrifuged for a further 10 minutes at 3000 rpm to remove cell debris. The supernatant was then stirred on ice. Solid bovine serum albumin (BSA, Sigma) was added to a final concentration of 5 mg/ml. The BSA was added to bind contaminating proteases which might otherwise degrade the procollagen (Mould, personal communication). Inhibitor stock solution (10x) was added to a final concentration of 0.1 M Tris-HCl/25 mM EDTA/10 mM N-ethylmaleimide (NEM)/1 mM phenylmethylsulphonyl-fluoride (PMSF) pH 7.4. Polyethylene glycol 4000 (PEG 4000, 25% in 0.15 M NaCl) was added to give a final concentration of 5% (Mould, personal communication). PEG-4000 at a final concentration of 5-10% has been shown to precipitate virtually all the procollagen in solution in tissue culture medium (Ramshaw, Bateman and Cole, 1984). The solution was left, unstirred, in the cold room at 4°C overnight. All subsequent procedures were carried out at 4°C.

The solution was centrifuged at 15,000 g in a J2-20 centrifuge (Beckman) for 30 minutes and the supernatant discarded. The pellet was washed in 100 ml 10 mM Tris-HCl pH 7.4 to remove PEG and centrifuged again at 15,000 g for 30 minutes. The pellet from this centrifugation was redissolved with stirring in approximately 15 ml 0.1 M Tris-HCl/0.4 M NaCl pH 7.4 (storage buffer) for 4 hours. After this time the resuspended pellet was centrifuged at 27,000 g for 45 minutes and the supernatant collected. The supernatant was dialyzed against 1000 ml 50 mM Tris-HCl/2M urea/10 mM NaCl/2.5 mM EDTA pH 7.8 (20°C) (DEAE start

buffer), with one change (Gerard and Mitchell, 1979).

2.1.4 Ion-exchange chromatography on DEAE-Sephacel

A column of DEAE-Sephacel (1.6 x 10 cm) was prepared. The column was packed at a flow rate of 100 ml/hour and equilibrated with DEAE start buffer. The dialyzate (32 ml) was then applied to the column at a flow rate of 20 ml/hour. The column was then washed with two column volumes of the starting buffer to remove unbound material. After sample adsorption, a gradient of total volume 400 ml was applied of 10 - 310 mM NaCl in DEAE start buffer. The fraction size was 8 ml and 40 fractions were collected. The fractions were assayed for ^{3}H-labelled protein by scintillation counting. Samples (50 μl) were added to 3.6 ml Cocktail T + 350 μl water (to give 10% aqueous phase) and counted (Packard Tri-Carb Liquid Scintillation Spectrometer). Peak fractions were pooled (a total of approximately 60 ml) and dialyzed against 0.1 M Tris-HCl/0.4 M sodium chloride pH 7.4 (20°C), with 2 changes.

After dialysis, the pooled fractions were concentrated using an ultrafiltration cell (Amicon) with a YM30 membrane (molecular weight exclusion limit 30,000 daltons). After concentration of the procollagen solution to a volume of 1 ml, the pressure source was removed and the concentrate was stirred for two hours. The solution was stirred in order to remove procollagen which had become bound to the YM30 membrane. Samples (10 μl) were taken for scintillation counting during this time to ensure that the sample was stirred for a sufficient length of time to release the procollagen from the membrane. The concentrated procollagen solution was collected and the ultrafiltration chamber was washed with 0.5 ml storage buffer, stirring for 2 hours. The wash was added to the concentrated procollagen solution, to give a final concentrated volume of 1.5 ml.

The procollagen solution contained approximately 7000

counts/μl. By hydroxyproline analysis (Section 2.3.1) yields of type I procollagen from the tendons of 30 dozen embryos were typically 1-1.5 mg, with a specific activity of approximately 10,500 counts/μg. The concentrated procollagen solution was stored at -70°C.

2.2 Purification of Type I Procollagen From Cornea

The method of section 2.1 was used successfully to purify type I procollagen from 17 day embryonic chick corneas (Kao, 1985).

Chick embryos at 17 days post-fertilization were decapitated. Using forceps to enlarge the eyelid opening, the forceps were inserted into the eye at the edge of the cornea and the corneas were removed by grasping them firmly with the forceps and pulling gently. The corneas were easily removed free of associated blood vessels or other tissues. Corneas isolated in this way were washed in HBSS (containing 1% PS) by centrifugation (50 ml tubes, MSE bench top centrifuge) for 2 minutes at low speed. HBSS in the supernatant and then fresh HBSS was added and this washing procedure was repeated twice.

The yield of corneas from 30 dozen embryos was about 7g. Digestion of the tissues was carried out using the same proportions of collagenase and trypsin as for tendon (Section 2.1.2). Digestion of corneas using this method took longer to complete than digestion of tendons, generally 1.5 hours. A typical yield of cells from a digest of corneas from 30 dozen embryos was 9 x 10^8 as determined by haemocytometry.

Labelling with L-5-^{3}H proline (1 μCi/ml; 37 kBq/ml) was carried out for 5 hours, when the culture was stopped. The corneal procollagen was then purified in the same way as the tendon procollagen.

The corneal type I procollagen was dialyzed against storage buffer and concentrated by ultrafiltration as

described (Section 2.1.4). From 30 dozen embryos typical yields of corneal type I procollagen were 500 μg with a specific activity of 8,500 counts/μg.

2.3 Characterization of Type I Procollagen and the Products of Procollagen Processing

2.3.1 Hydroxyproline Assay

The presence of large amounts of hydroxyproline in collagen compared with other proteins provides a marker which can be used to characterize and quantify collagenous proteins. The method used was that of Woessner (1961). Since hydroxyproline is peptide bound, it must be released from the protein for analysis by hydrolysis. After hydrolysis, the free hydroxyproline can be assayed using a method which involves the oxidation of the hypro to pyrrole by chloramine T, and then the formation of a chromophore by reaction with p-dimethylaminobenzaldehyde (p-DMEB, Ehrlich's reagent) (Figure 2.1). Proline is converted to Δ'pyrroline which is not converted to a chromogen by p-DMEB (Peterkofsky and Prockop, 1962).

Duplicate samples of tendon and corneal procollagen were taken for hydrolysis. The sample volume was made up to 1 ml with water and sufficient concentrated hydrochloric acid was added to produce a final concentration of 6 M (1.1 ml). The hydrolysis was carried out for 16 hours at 116°C in a Pyrex tube with a screw cap lined with Teflon, which was screwed on tightly to prevent evaporation of the solvent. After hydrolysis, the sample was evaporated to dryness using a Speed-Vac concentrator (Savant). Distilled water (1 ml) was then added to the samples. A series of standards of hydroxyproline (0-2.5 μg) were prepared in water and treated in the same way as the hydrolysed procollagen samples. Aliquots of 0.5 ml chloramine T solution (0.05 M chloramine T in 20% water,

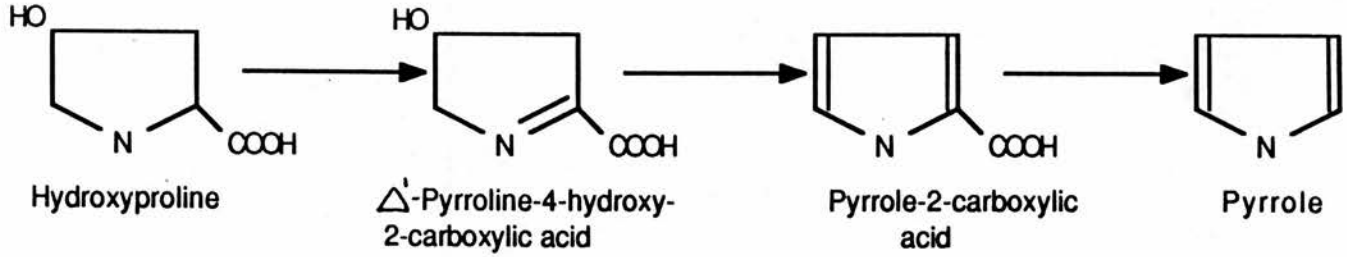

Figure 2.1 Reaction scheme showing the conversion of hydroxyproline to a chromogen in the assay of Woessner (1961). After Prockop and Udenfriend, 1960.

30% 2-methoxyethanol, 50% buffer) was added to each tube at 10 second intervals. The buffer was 0.24 M citric acid/0.21 M acetic acid/0.9 M sodium acetate trihydrate/ 0.85 M sodium hydroxide pH 6.0, stored under toluene. The tubes were shaken to ensure complete mixing, and allowed to stand for 20 minutes at room temperature. Perchloric acid (0.5 ml of a 3.15 M stock) was then added to each tube at 10 second time intervals. The perchloric acid stops the oxidation. Complete termination of the oxidation step was ensured by mixing the contents of the tube and leaving the tubes to stand for 5 minutes. Ehrlich's reagent (0.5 ml of 20% p-dimethylaminobenzaldehyde in 2-methoxyethanol) was added to each tube, and the contents of each tube were mixed until no schlieren pattern was seen. The chromophore complex was developed at 60°C for 20 minutes. This is the optimum time required for colour development (Woessner, 1961). After 20 minutes had elapsed the tubes were placed in cold water to stop the reaction. When the tubes were cold, the absorbance was read at 557 nm.

The amounts of hypro can then be determined from the hypro standard curve (Figure 2.2). As there are 85 residues/1000 in type I procollagen (Fiedler-Nagy et al, 1981) it can be assumed that 7.6% of type I procollagen is hydroxyproline, and therefore it is possible to determine the amount of procollagen in the sample, and the specific radioactivity of the procollagen.

$$\mu g \text{ procollagen in n } \mu l = \mu g \text{ Hypro} \times 100/7.6$$

Hence, μg procollagen/ml

$$= \mu g \text{ procollagen in n } \mu l \times 1000/n$$

Using this calculation, it was determined that the final concentrations of purified tendon and corneal procollagen were typically 700 μg/ml and 300 μg/ml respectively.

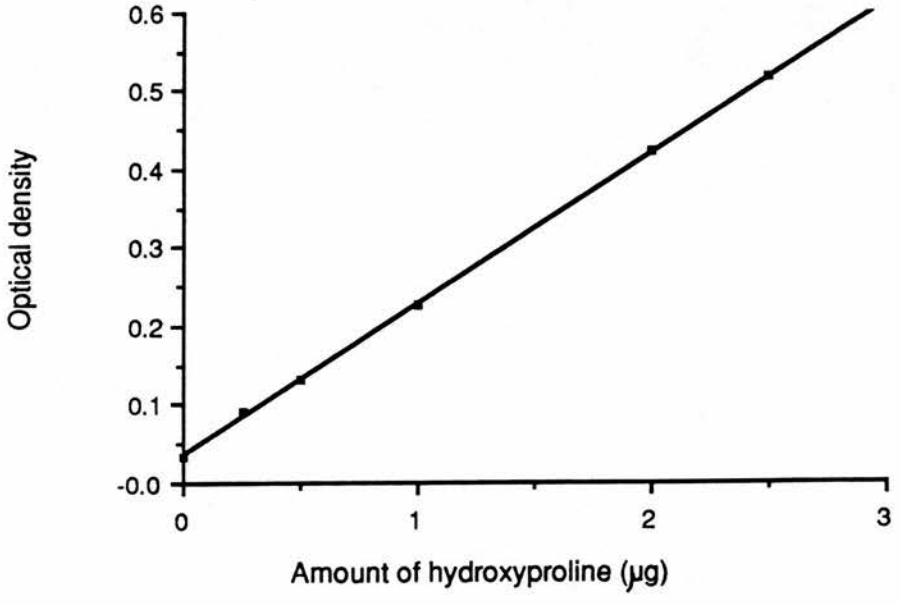

Figure 2.2 Typical curve showing the absorbance of L-hydroxyproline standards (0-2.5 µg) at 557 nm when treated in the assay of Woessner (1961).

2.3.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The molecular weights of most proteins can be determined by measuring their mobilities in polyacrylamide gels containing the anionic detergent sodium dodecyl sulphate (SDS). When samples containing 2% SDS and 3% β-mercaptoethanol are heated at 100°C for 3 minutes, the proteins bind SDS and denature. Disulphide linkages are broken by the mercaptoethanol and the protein-SDS complexes assume a random coil configuration. The amount of SDS bound/weight of protein is constant, and therefore all the protein chains have an identical charge:mass ratio. However, collagenous proteins behave anomalously on SDS-PAGE in comparison with most globular proteins, and a simple relationship between mobility and molecular weight no longer applies (see Furthmayr and Timpl, 1971; Butkowski, Noelken and Hudson, 1982). The behaviour could be due to localized conformation effects resulting from restricted rotation of polypeptide chains about imino acid residues (Furthmayr and Timpl, 1971). Using this method it is possible to separate the proα1(I) and proα2(I) chains and also any degradation products of the chains.

Samples were analyzed by discontinuous SDS-PAGE. The composition of the separating gel was 6% acrylamide/0.16% N,N'-methylenebisacrylamide/0.375 M Tris-HCl/0.2% SDS/0.05% ammonium persulphate (AMPS) pH 8.8, with 0.0005% N,N.N',N'-tetramethylethylenediamine (TEMED) added to catalyze polymerization. The separating gel measured 12 cm x 16 cm x 1.5 mm. The stacking gel (4 cm x 16 cm x 1.5 mm) was composed of 4.5% acrylamide/0.12% methylenebis-acrylamide/0.2% SDS/0.125 M Tris-HCl/0.05% AMPS pH 6.8, again with TEMED to catalyze the polymerization.

Samples of corneal and tendon procollagen containing 10,000 cpm) were analyzed by electrophoresis. To each sample, one-fifth the volume of 5 x sample buffer was added (0.125 M Tris-HCl/50% glycerol/2% SDS/15%

β-mercaptoethanol/0.05% bromophenol blue), and the samples were boiled for three minutes. The samples were applied to the stacking gel using a microsyringe (Kloen). The gels were run at a constant current of 40 mA in 0.025 M Tris/0.192 M glycine/0.1% SDS pH 8.3 until the dye front was 1 cm from the bottom of the gel. The gel was then removed from the plates and processed for fluorography.

2.3.3 Fluorography

The positions of the radiolabelled proteins were visualized by fluorography. In the absence of a fluor, radioactive emissions can activate individual silver halide grains in the photographic emulsion to render them susceptible to conversion into metallic silver using a reducing agent (developer). But the β particles produced by ^{3}H decay are of low energy and are detected with less than 100% efficiency. To increase the efficiency of detection, a fluor, 2,5-diphenyloxazole (PPO), is used (Bonner and Laskey, 1974), enabling the β particles to interact more efficiently with the silver halide crystals.

Gels were fixed for 30 minutes in glacial acetic acid (Skinner and Griswold, 1983). As PPO is insoluble in water, the water must be removed from the gel before impregnation with the PPO (Bonner and Laskey, 1974). The gel was then transferred to a solution of 20% PPO in glacial acetic acid and agitated gently for 45 minutes on a reciprocal shaker (Denley). This percentage of PPO in glacial acetic acid gives optimal sensitivity (Bonner and Laskey, 1974). After this time excess 20% PPO was removed and water was added to precipitate out the PPO in the gel. The gel was washed in several changes of tap water and then finally placed in distilled water containing a few drops of glycerol. The gel was then shaken for a minimum time of 45 minutes before being covered with plastic cling film and dried down on a gel dryer (LKB). The presence of glycerol prevents the gel from cracking whilst being dried

(Skinner and Griswold, 1983). After drying, the gel was exposed to X-ray film (XAR 5 X-OMAT, Kodak) at -70°C. The low temperature increases the efficiency of fluorography, possibly by decreasing the rate of a back-reaction of a single silver atom to a silver ion, hence increasing the rate of formation of a stable pair of silver atoms (Laskey and Mills, 1975). After exposure the film was developed by immersion in hydroquinone-sodium carbonate developer (D-19, Kodak), then placed in a stop bath for 30 seconds (24% acetic acid, Kodak) and finally fixed in inorganic thiosulphate (FX-40, Kodak).

2.3.4 Prefogging of X-ray film

It is not possible to calculate accurately the distribution of radioactivity in the bands by densitometry without prior pre-fogging of the X-ray film, as the absorbance of the fluorographic image is not proportional to the amount of radioactivity in the gel, or the time of exposure (Laskey and Mills, 1975). This can be explained by the fact that each silver halide grain requires about 5 photons of light to reach a 50% probability of being developed by the photographic solutions. Each photon produces a single silver atom, but this is unstable and can revert back to a silver ion by thermal decomposition. This decomposition can only be stabilized by the production of a second silver atom which prevents the back reaction. From this point onwards, growth of the image is stable. However, unless the initial photons are produced faster than 1 photon/crystal/second (half life for the back reaction), a latent image will have a greatly reduced chance of accumulating and low intensities will be under-represented (Laskey and Mills, 1975). Thus, pre-exposing the film to a very short flash of light (< 1 s) should enable the stable pair of silver atoms to be preformed before exposing the film to the gel. This will have the effect of increasing the sensitivity of the film to small

amounts of radioactivity, since the initiation of image formation will no longer be dependent on the formation of a stable pair of silver atoms. Also, below the saturation limit of the film the absorbance will be linearly related to the radioactivity, since all the photons produced by the sample will have an equal probability of contributing to the growth of the latent image (Laskey and Mills, 1975).

Accordingly, films were pre-exposed to a short flash from a photographic flash unit (Kodak). A Wratten no. 21 coloured filter and a piece of Whatman no. 1 filter paper were taped to the unit to reduce the light output and diffuse the light, respectively. The film (Kodak XAR 5 X-OMAT) was on a background of white Whatman no. 1 filter paper and the surface nearest the light source was applied to the dried gel (Laskey and Mills, 1975). Pre-exposure of the film was carried out in the dark, with no safelights. The intensity of the flash could be varied by altering the distance of the film from the flash unit. Positioning the flash unit at a distance of 35 cm above the film gave an increase in background "fog" 0.15 OD units above the background level of unexposed film. This increment in background fog absorbance results in a completely linear relationship between photographic image absorbance and amount of radioactivity (Laskey and Mills, 1975) for all bands of intensity less than 1.5 OD units (Laskey and Mills, 1975).

2.3.5 Densitometry

All fluorograms were scanned using the Chromoscan 3 (Joyce Loebl). The Chromoscan 3 was linked to an IBM-compatible DCS microcomputer and operated using the Chromoscan 3 External Data Analysis package (Joyce Loebl).

The basis of densitometry is the Beer-Lambert law which relates the amount of a chromophore to the measured

extinction, E,

$$E = \log_{10}(I_o/I) = kcl$$

where I and I_o are the intensities of transmitted and incident light respectively; l=path length of the light through the sample; k is the extinction coefficient for the particular chromophore at a distinct wavelength (eg Goldstein, 1971; Chayen, 1978, 1984; Bitensky, 1980). However, this is not the situation with fluorograms. The loss of light caused by the silver grains in an autoradiographic emulsion is not strictly comparable to the absorption of light by a chromophore (Goldstein, 1971; Bitensky, 1980).

As the bands on fluorograms are not homogeneous, the scanning densitometer optically "divides" the area to be scanned into many areas, the extinction of which is measured separately. The results from all these areas are summed, giving a total integrated absorbance (Chayen, 1978, 1984; Bitensky, 1980). The Chromoscan 3 scans the fluorogram with a slit, and the transmittance is approximately proportional to the area of emulsion not covered by the grains (Goldstein, 1971).

The area occupied by the silver grains is linearly related to the apparent absorption (Bitensky, 1980) and thus the intensities of different bands in a fluorogram can be related to each other.

The Chromoscan 3 was operated in transmission mode with an aperture of 0.3 x 5 mm. The light source was a 100W tungsten halogen lamp, and measurements were carried out at a wavelength of 530 nm. Data were obtained every 50 μm down the length of the gel. The scanning rate was 30 mm/sec. The density data was digitized to 8 bits (i.e 256 grey levels).

Before calculating integrated peak intensities, a background correction was made, described as a "rubber band" method. This is analogous to stretching a rubber band around the lowest trough point from the starting

point to the ending point. The values of the integrals of the peaks were obtained by subtraction of the background.

2.4 *In vitro* Fibrillogenesis

During this study, purified tendon and corneal type I procollagens were incubated with purified procollagen N- and C-proteinases at 37°C. This system has been previously used to form fibrils from chick tendon procollagen (Miyahara *et al*, 1982, 1984) and from human skin type I procollagen (Kadler, Hojima and Prockop, 1987), but this is the first time that this system has been used to form fibrils *in vitro* from corneal type I procollagen. Fibril formation *de novo* from tendon and corneal type I procollagen were compared by incubating purified tendon and corneal procollagens with the purified N- and C-proteinases.

2.4.1 Assays for the Processing Enzymes

Two methods can be used to assay the procollagen N- and C-proteinases. One method is to use SDS-PAGE to examine the cleavage products which are then quantitated by densitometry. The other method involves precipitation of the uncleaved procollagen and the partially cleaved intermediates with ethanol, leaving the cleaved N- or C-propeptides in the supernatant. Results from the ethanol precipitation assay are obtained more quickly than from the gel assay, because the gel has to be dried, processed for fluorography and a film exposed and developed. Thus the precipitation assay is known as a "rapid assay". Both assays were used in the present study.

2.4.2 Assays for Procollagen C-Proteinase

The method for assaying C-proteinase in a rapid assay was based on those developed by Kessler and Goldberg

(1978) and Hojima, van der Rest and Prockop (1985). The assay is based on the observation that the released C-propeptide is soluble in 25% ethanol, whereas uncleaved procollagen and the procollagen intermediates are not (Kessler and Goldberg, 1978). Each assay tube contained 0.6 μg ^{3}H-proline-labelled procollagen of known specific activity (10,500 cpm/μg for the tendon type I procollagen; 8,500 cpm/μg for the corneal type I procollagen). The substrate procollagen was in a buffer of 0.1 M Tris-HCl/0.4 M NaCl pH 7.4 (20°C) and this was diluted with an equal volume of "procollagen dilution solution" (10 mM $CaCl_2$/0.01% Brij/0.02% NaN_3). The procollagen substrate was then further diluted with "assay buffer" (50 mM Tris-HCl/150 mM NaCl/5 mM $CaCl_2$/0.05% Brij/0.01% NaN_3 pH 7.7 at room temperature) to give the final concentration of substrate required (60 μg/ml). The assay was carried out in microcentrifuge tubes, each containing a final volume of 50 μl (i.e. each tube contained 0.6 μg procollagen/50 μl). Diluted procollagen substrate (60 μg/ml; 10 μl) was added to each reaction tube. The remaining 40 μl consisted of C-proteinase at a suitable dilution in assay buffer. Control tubes consisted of 10 μl substrate + 40 μl assay buffer. The assay was carried out at 35°C for 4 hours. After this time the tubes were immediately placed on ice and 50 μl "stop" buffer (50 mM Tris-HCl/150 mM NaCl/30 mM EDTA/3mg/ml fibrinogen pH 7.5) were added to each tube in order to terminate the assay, and the tubes were vortexed. The EDTA was necessary to inhibit the enzyme; a concentration of 5mM is known to be completely effective (Hojima, van der Rest and Prockop, 1985). The fibrinogen acted as a carrier for the precipitate which formed after the addition of ethanol (50 μl of 75% ethanol at -20°C). The tubes were then thoroughly vortexed to ensure complete mixing. The tubes were then left on ice for 1 hour to allow precipitation to proceed. The tubes were then centrifuged at 11,600 g in a MSE MicroCentaur at 4°C for 15 minutes. Half the volume of the supernatant (75 μl) was

transferred to a scintillation vial. Water (325 µl) and Scintran Cocktail T (3.6 ml) were added to each vial, and the radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer.

The activity of the enzyme could then be calculated. From amino acid sequence data, it was calculated that 4% of the total radioactivity (pro + hypro) is present in the C-propeptide (Galloway, 1982). The specific activity of the tendon procollagen substrate was 10,500 counts/µg. One unit of enzyme activity is defined as that amount of enzyme which cleaves 1 µg procollagen in 1 hour at 35°C (Hojima, van der Rest and Prockop, 1985).

Hence, 1 unit = $10{,}500 \times \frac{4}{100} \times \frac{75}{150}$ cpm released per hour

= 210 cpm released /hour

Therefore,

units/ml =

$$\frac{\text{net cpm above background}}{210 \times t} \times \frac{1000}{40} \times \text{dilution factor}$$

where t = duration of assay

For the activity of the enzyme to be assayed by SDS-PAGE, a 50 µl incubation mixture was prepared as above and incubated at 35°C. At the end of the assay period, "stop" solution was added as above, but no fibrinogen was present in the stop solution. Ethanol was not added. SDS-PAGE sample buffer (25µl) was added to each tube (2.3.2). The tubes were then boiled for 3 minutes and loaded on a 5% SDS-polyacrylamide slab gel. Electrophoresis was carried out as described in Section 2.3.2. The gels were processed for fluorography as described (Section 2.3.3). The extent of cleavage could then be assessed by densitometry.

2.4.3 Rapid Assay for Procollagen N-Proteinase

The rapid assay employed was very similar to the one described above for the C-proteinase (Section 2.4.2).

Dilutions of the substrate were prepared as above. The same assay volume was employed (50 µl), with 10 µl of substrate and 40 µl of the diluted N-proteinase (diluted in assay buffer). The reaction mixture was incubated at 35°C for 4 hours and stopped as described, except that the stop buffer did not contain fibrinogen. Ethanol (50 µl of 81% ethanol stored at -20°C) was added, resulting in a final concentration of 27% (Nusgens and Lapière, 1979).

After the ethanol precipitation had proceeded for 1 hour, the assay tubes were centrifuged at 11,600 g for 15 minutes. When assaying N-proteinase, a pellet was not visible after centrifugation. Hence the supernatant from the centrifugation needed to be collected carefully to avoid removing procollagen and pC-collagen which did not sediment completely. Samples were treated for scintillation counting as described (Section 2.4.2).

It was calculated that 10 % of the ^{3}H-proline-associated radioactivity is present in the N-propeptide (Galloway, 1982). The specific radioactivity of the tendon procollagen was 10,500 counts/µg. One unit of N-proteinase activity is the amount of enzyme required to cleave 1 µg of procollagen in 1 hour at 35°C.

Then,

$$1 \text{ unit} = 10{,}500 \times \frac{10}{100} \times \frac{75}{150}$$

$$= \text{n cpm released per hour} = 525$$

Therefore,

$$\text{Units/ml} = \frac{\text{net cpm above background}}{525 \times t} \times \frac{1000}{40} \times \text{dilution factor}$$

The gel assay for procollagen N-proteinase was carried out exactly as described for the C-proteinase (Section 2.4.2).

2.4.4 *In Vitro* Fibril Formation

It has been shown that incubation of procollagen and the processing enzymes in a physiological buffer at 37°C results in the formation of D-periodic fibrils (Miyahara *et al*, 1982; 1984; Kadler, Hojima and Prockop, 1987). In the work detailed here, the buffer devised by Kadler, Hojima and Prockop (1987) was used (i.e. 117 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/1.03 mM NaH_2HPO_4/0.81 mM $MgSO_4$/0.01% NaN_3/ 20 mM $NaHCO_3$ pH 7.3). The procollagen and enzymes used were dialyzed against this buffer prior to mixing. After dialysis, rapid assays were carried out on the N-proteinase and C-proteinase respectively to determine any possible losses of activity on dialysis.

A relatively high concentration of procollagen was used (120 μg/ml) for fibril formation. Appropriate amounts of enzymes and substrates were mixed at 4°C; the tubes were gassed with 95% O_2/5% CO_2 and then incubated at 37°C for 24 hours. Samples of corneal and tendon procollagen were incubated in triplicate; in addition, samples were also incubated for subsequent analysis by SDS-PAGE.

After incubation the samples for SDS-PAGE were centrifuged at high speed (11,600 g) in the microcentrifuge for 5 minutes at room temperature. The supernatant was carefully removed with a Gilson micro-pipette. Then, 0.5 M Tris-HCl/125 mM EDTA/10% SDS (12.5 μl) was added and the tubes whirlimixed. Fibril formation buffer (50 μl) was added to the pellets, then 12.5 μl of 0.5 M Tris/125 mM EDTA/10% SDS, and the tubes were whirlimixed.

The supernatants and pellets were heated immediately at 100°C for 3 minutes. One-quarter of the total sample volume of sample buffer was added before SDS-PAGE. The samples were boiled for three minutes and analyzed on 6% acrylamide gels (Section 2.3.2).

The samples which were to be examined by electron microscopy were centrifuged for 5 minutes at 11,600 g. The

supernatants were carefully removed and then fixative (100 µl of 4% formaldehyde in 0.1 M cacodylate pH 7.4) was added. When fixation was complete (at least 1 hour at room temperature) the formaldehyde solution was removed and approximately 200 µl of 0.5% agarose were then added.

2.4.5 Electron Microscopy

Samples were fixed and agarose embedded in Edinburgh and then processed for electron microscopy (EM) by Mrs C Cummings and Ms K Greenwood (University of Manchester).

The sample pellets embedded in agarose were stained with 1% phosphotungstic acid (PTA) for 1 hour and then in 1% uranyl acetate (UA) for 1 hour. PTA binds to positive ions such as the ε-amino group of lysine, hydroxylysine, arginine and histidine. UA is a weak electrolyte in aqueous solution, and exists not only as uranyl ions but also as anionic and cationic complexes. By using a combination of PTA and UA all the charged residues should be bound by heavy metal (Chapman and Hulmes, 1984).

After staining, the samples were dehydrated in a series of graded ethanol solutions, from 50% ethanol, through 70%, 80%, 90% and 95% ethanol (10 minutes each). The samples were then transferred to 100% ethanol for 15 minutes and this step was repeated twice. The samples were then transferred to propylene oxide, for 2 x 15 minutes. The propylene oxide step is necessary because the ethanol does not mix readily with the resin, so the ethanol must be replaced. The specimens were then embedded in Araldite. An Araldite mixture was prepared, consisting of 20 ml Araldite/20 ml dodecyl succinic anhydride/0.8 ml benzyldimethylamine. The specimens were then infiltrated in 50% propylene oxide/50% Araldite mixture for 30 minutes. They were then transferred into 100% Araldite mixture and left overnight. This Araldite was then replaced with fresh mixture and infiltrated for 6 hours to remove any remaining propylene oxide. The specimens were

then transferred to an embedding mould and polymerized at 60°C for 48 hours.

Thin sections were cut on an ultramicrotome (LKB Ultratome) using a diamond knife. The sections were then picked up on a carbon collodion coated copper grids and if necessary further stained with 1% PTA/1% UA.

The specimens were then examined using a JEOL 1200 EX electron microscope.

2.5 Lectin Blotting

The lectin chosen for detection of the hydroxylysylglycosides was viscumin, the component in mistletoe responsible for cytotoxicity (Olsnes et al, 1982). The lectin exerts its toxic effects by binding to the 60S subunit of ribosomes (Olsnes et al, 1982). Viscumin seems to bind to cell surface receptors containing terminal galactose residues; glucose and mannose do not prevent the action of viscumin (Stirpe et al, 1982). Thus, this lectin was chosen as it will hopefully bind to the galactosyl residues on the corneal and tendon procollagens. The structure of the disaccharide glucosylgalactosyl-O-lysine is shown in Figure 2.3. As well as galactosyl and glucosylgalactosyl residues, type I procollagen molecules also contain a high mannose oligosaccharide attached to an asn residue in the C-propeptide. It was not clear if the lectin might bind to these residues or if it was selective for galactose. Since it was hoped that it might be possible to detect differences in the levels of galactose between the two procollagens, it was decided to remove the propeptides in order to eliminate any binding due to the presence of sugar on the propeptides. This was accomplished by treatment of the corneal and tendon type I procollagens with pepsin.

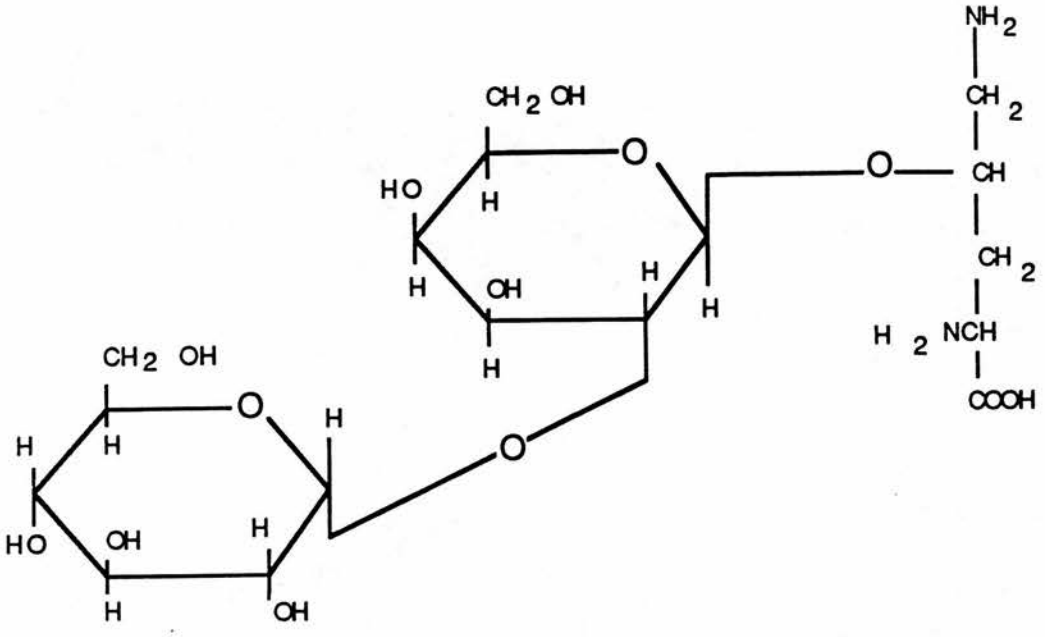

Figure 2.3 Structure of the disaccharide 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine.

2.5.1 Pepsinization of the Procollagens

The method used was that of Helseth and Veis (1981). Aliquots of tendon and corneal type I procollagen were dialyzed overnight against 0.01 M acetic acid at 4°C. After dialysis, pepsin was added in an enzyme:substrate weight ratio of 1:10. Pepsin digestion was carried out for 24 hours at room temperature (Helseth and Veis, 1981).

It was desirable for the pepsinized procollagen samples to be dissolved in a Tris/SDS buffer, not 0.01 M acetic acid, for SDS-PAGE. This was achieved by adding 30% NaCl in 0.01 M acetic acid to a final concentration of 10% sodium chloride. This concentration of sodium chloride was used to precipitate out the pepsinized protein overnight at 4°C. The samples were then centrifuged at 11,600 g for 15 minutes (MSE) in order to precipitate the pepsinized proteins. The supernatants were carefully removed and the precipitates were redissolved in 0.125 M Tris-HCl/2% SDS pH 6.8. A sample of rat tail tendon collagen in 0.1 M acetic acid was also subjected to precipitation with 10% NaCl, centrifugation and redissolution in 0.125 M Tris-HCl/2% SDS pH 6.8. Efficiency of precipitation was determined where appropriate by scintillation counting of supernatants.

2.5.2 Lectin blotting

The pepsinized samples of tendon and corneal type I procollagen were prepared for electrophoresis as described in Section 2.3.2. Samples of rat tail tendon collagen (1 mg/ml, provided by Dr D R Shackleton) were subjected to precipitation with 10% NaCl and resuspension of the pellet with 0.125 M Tris-HCl/2% SDS pH 6.8 as described above. The rat tail tendon collagen had been prepared by neutral salt extraction/acid-salt precipitation and collagen extracted under such conditions is known to be glycosylated (e.g. Schofield, Freeman and Jackson, 1971).

Thus, running such samples on SDS-PAGE should be a positive control to check that the lectin will bind to the collagen. Samples of rat tail tendon collagen (20 μg, 50 μg and 100 μg) were taken for electrophoresis. In addition to these collagen standards and the pepsinized tendon and corneal procollagen samples, molecular weight markers were electrophoresed (supplied by Dr D K Apps). The molecular weight markers should be a negative control. Biotinylated molecular weight markers were also provided by Dr D K Apps.

The samples were electrophoresed under reducing conditions as described in Section 2.3.2. After electrophoresis, the gels were electrically transblotted onto nitrocellulose (EC corporation, St Petersburg; pore size 0.45 μm). A sheet of nitrocellulose was briefly wetted by floating it on the surface of the transblotting buffer. Two scouring pads were immersed in the transblotting buffer. The gel was laid on the surface of the scourers and all air bubbles were removed. A sheet of wetted nitrocellulose was then overlaid on the gel and air bubbles were again removed. A sheet of Whatman 3 mm paper was soaked in the buffer and overlaid on the nitrocellulose. Another gel was laid on top of this and this gel was overlaid with another sheet of nitrocellulose as described. Another piece of filter paper was placed on top of the nitrocellulose and three soaked scouring pads were placed on top of this (Towbin, Staehelin and Gordon, 1979). The whole 'sandwich' assembly was enclosed in a perforated stainless steel support and this ensured that the gels were firmly and evenly pressed against the nitrocellulose.

Many proteins adsorb to nitrocellulose filters (eg Freifelder, 1982). Towbin, Staehelin and Gordon (1979) found that application of an electric field perpendicular to slab gels results in a replica of the protein band pattern on the nitrocellulose sheet. The proteins are transferred electrically from a negative to a positive

direction onto the nitrocellulose (Towbin, Staehelin and Gordon, 1979).

The gels were transblotted at 1 ampère for 2 hours. The transblotting buffer was 20 mM NaH_2PO_4/0.02% SDS/20% methanol (Apps, Phillips and Purves, 1985). The methanol seems to increase the binding capacity of nitrocellulose membranes.

While the proteins were being transferred to nitrocellulose, the lectin was biotinylated. The viscumin (5 mg) was dissolved in 1 ml of water. 0.2 ml of this was transferred to another tube and 20 μl 1 M bicine buffer pH 8.3 was added. Bicine is used because it contains no primary amine groups, and therefore does not interfere with the biotinylation. N-hydroxysuccinimidobiotin (NHS-biotin) (10 μl) of a 20 mM stock (7 mg/ml in dimethyl-sulphoxide, DMSO) was added to the lectin-bicine solution. Biotin contains a carboxylic acid group and the NHS-group is ester O-linked to biotin. When NHS-biotin is dissolved in DMSO, the biotin will react with primary amines, notably the ε-amino group of lysine residues. The NHS-biotin was incubated with the lectin for one hour at room temperature.

At the end of the period of incubation the lectin-biotin complex was separated from the free lectin as follows. First, a hole was pierced in the bottom of an Eppendorf tube and plugged with glass wool. The remainder of the Eppendorf tube was filled with a suspension of BioGel P6DG which had been equilibrated with phosphate-buffered saline (0.15 M NaCl/0.05 M Na_2PO_4 pH 7.4, PBS). Then excess PBS was removed and the lectin-biotin solution was placed on the surface of the 'mini-column'. The 'mini- column' was placed on a test tube and the assembly was centrifuged at 1400 rpm in a bench centrifuge (MSE) for 1 minute. The liquid recovered in the bottom of the test tube consisted of the lectin-biotin complex; as this is of high molecular weight it does not enter the beads and passes straight through the column. This method enables a quick and

effective separation of the lectin to be obtained, and was developed by Dr D K Apps.

After transfer of the proteins to nitrocellulose, the original SDS-gels were processed for fluorography as described (Section 2.3.3). The pieces of nitrocellulose were then incubated for 1 hour in 0.2 M Tris/1.5 M NaCl/0.18 M HCl (Tris/salt; pH 7.3 at room temperature) which had been diluted 10-fold. The diluted buffer contained 0.25% Tween-20 to block spare binding sites, bovine serum albumin (2 mg/ml, to protect the lectin from proteolysis) and the lectin diluted 100-fold (50 µl in 50 ml).

The lectin solution was left on the gels overnight . The solution was then poured off and the blots were washed in 10 x diluted Tris/salt solution (containing 0.02% Tween). Five washes, each of 10 minutes duration, were carried out (Towbin, Staehelin and Gordon, 1979). After the final wash, 10x diluted Tris/salt without Tween was added to the blots. This Tris/salt contained ^{125}I-streptavidin at an activity of 10 counts/second/µl. 15 l ^{125}I-streptavidin was present in 100 ml diluted Tris/salt to give this activity.

The nitrocellulose blots were incubated with the ^{125}I-streptavidin for 1 hour, and were then washed for 50 minutes (4 changes) in 10x diluted Tris/salt without Tween. The blots were placed on filter paper and dried in air overnight. They were then exposed to X-ray film (XAR 5 X-OMAT, Kodak) at -70°C.

CHAPTER 3

DEVELOPMENT OF THE PULSE-CHASE PROTOCOL

3.1 Introduction to the Present Study

This chapter describes the development of a pulse-chase protocol which would enable the rate constants of processing to be determined. For the present study, the rate constants were defined as k_1 (procollagen to pC-collagen), k_2 (procollagen to pN-collagen), k_3 (pC-collagen to collagen) and k_4 (pN-collagen to collagen), shown in Figure 3.1.

3.2 Materials

Fertilised hen eggs (J strain) were obtained from the Poultry Research Centre, Roslin, Midlothian, and incubated at 37°C (Western incubator) in a humid atmosphere until required. Experiments were carried out at 12 days, 14 days, 17 days and 19 days post-fertilization (stages 38, 40, 43 and 45; Hamburger and Hamilton, 1951; Hamilton, 1952). L-5-^{3}H proline (30 Ci/mmol; 1.11 TBq/mmol) was obtained from Amersham. L-4-^{3}H hydroxyproline (5.3 Ci/mmol; 0.196 TBq/mmol) was obtained from New England Nuclear. Dowex AG50W-X8 was supplied by Bio-Rad. Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin (PS, 10000 units/ml, 10000 μg/ml) and Minimal Essential Medium (MEM) were all obtained from Gibco (for composition of media, see Appendix 1). Phenylmethylsulphonylfluoride (PMSF), N-ethyl maleimide (NEM), cycloheximide, β-aminopropionitrile (BAPN) and Tris were obtained from Sigma. Cacodylic acid and paraformaldehyde were purchased from Agar Aids. All other chemicals were from BDH.

3.3 Use of Chick Embryos for the Experiments

Chick embryos were chosen for these studies for the following reasons:

a) they are readily available;

b) their development has been extensively studied, and

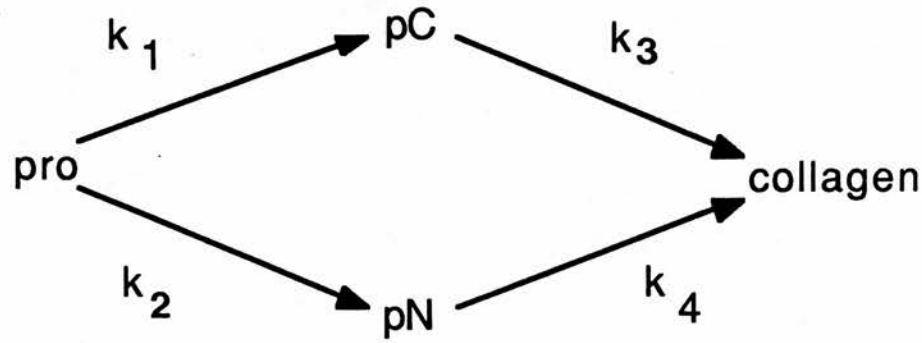

Figure 3.1 Routes of processing of procollagen showing the rate constants of the individual steps of processing k_1, k_2, k_3 and k_4.

therefore experiments can be carried out at well-defined developmental stages;

c) the embryo stage is a period of rapid growth and thus synthesis of connective tissue proteins is extensive.

3.3.1 Staging of Chick Embryos

Chick embryos at several different developmental stages were used for the pulse-chase experiments. As embryonic development involves rapid growth and hence rapid synthesis of proteins, it was decided that it would be interesting to compare the rates of procollagen processing and the fibril diameters at different developmental stages.

It was important that all the tissues used for each pulse-chase experiment were at the same developmental stage. It is not satisfactory to stage embryos with a classification based on chronological age, because enormous variations may occur in embryos, even if all the eggs in a setting are placed in the incubator at the same time (Hamburger and Hamilton, 1951). There may be genetic differences in the rate of development of different breeds; seasonal differences in viability and vigour of embryos; differences in the stage of development when incubation is started; differences in the lapse of time between laying and incubation; differences in the temperature of individual eggs when placed in the incubator, and the size of individual eggs (Hamburger and Hamilton, 1951). Hence, embryos were staged by measuring the length of the third toe from the tip to the middle of the metatarsal joint (Hamilton, 1952). After decapitation of the chick embryos, the legs were removed and placed on a Petri dish. The length of the third toe could be measured by placing the dish on a microscope stage, observing the leg at 10 x magnification, and measuring the toe length with a piece of 1 mm^2 ruled graph paper.

Despite the observations of Hamburger and Hamilton of

great variation in external form between eggs of the same setting, the differences in lengths of the third toe at any stage was never greater than 6.7%. The length of the third toe was also constant from batch to batch. Thus, all the embryos used in an experiment were at the same developmental stage.

The length of the third toe was determined for embryos at 12 days, 14 days and 17 days of embryonic development. These lengths were:

12 days = 6.53 +/- 0.24 mm
14 days = 9.15 +/- 0.22 mm
17 days = 14.5 +/- 0.47 mm

The lengths given by Hamilton (1952) are 8.4 +/- 0.3 mm (12 days); 12.7 +/- mm (14 days); and 18.6 +/- 0.8 mm (17 days). This is possibly due to genetic differences between the strains. Also, the difference between Hamilton's measurements and the current measurements is 30% at 12 days, 19% at 14 days and 13% at 17 days. The decrease in difference between the current measurements and the measurements of Hamilton may be accounted for by the fact that embryos at 12 days are quite small and thus greater errors in measurement may result from measuring smaller toes.

3.4 Development of the pulse-chase protocol

Initially, two alternative approaches were considered. Scheme I: pulse-labelling <u>in ovo</u> with ^{3}H-proline, and then chasing with unlabelled proline. Scheme II: removal of the tissues, pulse-labelling and chasing in organ culture. Scheme I seemed attractive as rate constants would be obtained approximating the <u>in vivo</u> situation, assuming ^{3}H-proline acts as a perfect tracer (section 3.6.3). However, scheme I was rejected in favour of scheme II because :

a) It would not be certain that the delivery of the label to the tissues would be constant (Moen, Rowe and

Palmiter, 1979) and larger amounts of radioactive label would be required;

b) There would be problems with replacement of the radioactive label with non-radioactive proline;

c) It would be difficult to dissect out the radioactive tissues and dispose of the radioactive animal waste safely.

Thus, it seemed simpler to remove the tissues of interest (scheme II). Although scheme II is not an ideal in vivo situation, it was sufficient for the purposes of these experiments; namely, to compare the rate constants of the individual steps k_1, k_2, k_3 and k_4 in tendons and corneas (Figure 3.1).

Previous pulse-chase systems to investigate the processing of type I procollagen include: intact chick calvaria at 18 days of development (Fessler, Morris and Fessler, 1975; Morris et al, 1975) and at 17 days of development (Davidson, McEneany and Bornstein, 1975); 17/18 day chick blood vessels (Fessler and Fessler, 1979; Fessler, Timpl and Fessler, 1981); rat periodontal ligament (Limeback and Sodek, 1979); 17-day chick embryo tendons (Uitto and Lichtenstein, 1976; Leung et al, 1979). Type I procollagen processing has also been studied in cell culture: mouse and human dermal fibroblasts (Limeback and Sodek, 1979); cultured bovine smooth muscle cells (Gerstenfeld et al, 1984); guinea pig fibroblasts (Sonohara et al, 1981); chick tendon fibroblasts (Uitto and Lichtenstein, 1976); human and bovine fibroblasts (Minor et al, 1986); chick calvarial osteoblasts (Gerstenfeld et al, 1988); and dermal fibroblast cultures (Bateman et al, 1986). In those cell culture studies where the time course of processing was followed, complete processing of type I procollagen took greater than 7 hours to occur, and in many cases processing was not complete after 2 days in culture. In contrast, in intact tissues type I procollagen processing was complete within 3 hours. The greater length of time taken for completion of

processing in cell cultures is possibly due to the dilution of procollagen and the processing enzymes that would occur in the large volume of medium used for cell culture which surrounds the cells (Uitto and Lichtenstein, 1976; Bateman et al, 1986). Intact tissues were used for these experiments as this is a more physiological system than cell culture.

3.4.1 Length of pulse

A pulse-chase method was used to determine the rate constants k_1, k_2, k_3 and k_4. In this protocol a "pulse" of ^{3}H-proline is administered for a short period of time. The radioactive tracer will ideally label only a single population of molecules whose fate can then be followed by removing the radioactive label and "chasing" with unlabelled proline. The original protocol used here is shown in Table 3.1.

There is a wide variation in the length of the pulse-labelling period used by previous investigators. Previous experiments on tendon used pulse times of 15 minutes (Leung et al, 1979) or 60 minutes (Uitto and Lichtenstein, 1976). Initially, a pulse time of 30 minutes was chosen. For the purposes of quantitation, it was desirable to have a pulse length such that procollagen was not processed until after the start of the chase period, so that all the radioactivity was in the proα1(I) and proα2(I) bands. After SDS-PAGE and fluorography of the protein extracts from the initial experiments, it could be seen on examination of the fluorograms that, using this criterion, a pulse time of 30 minutes was too long, as processing had already started to occur. This indicated that the secretion time must be less than 30 minutes after synthesis, as processing does not commence until the procollagen has been secreted to the extracellular space (Olsen and Prockop, 1974; Weinstock, 1974; Morris et al, 1975). Thus, the pulse time was reduced to 20 minutes, in

1. Semi-Sterile Technique

Autoclave dissection instruments, Eppendorf tubes. Dissect in sterile Petri dishes.

2. Dissection

Dissect out chick embryo tendons at 12, 14, 17 or 19 days of development in HBSS/1% PS. Measure length of third toe. Place one or two tendons in fixative (4% formaldehyde in 0.1 M cacodylate).

3. Preincubation of Tissues

Label Eppendorf tubes (duplicate samples): 0, 10, 15, 20, 30, 60, 90, 120. Add MEM (450 µl) to each tube, followed by 25 µl of freshly prepared 1 mg/ml ascorbate/1.28 mg/ml BAPN. Add tendons from 2 legs to tube 120. Gas the tube with 95% O_2/5% CO_2 and preincubate at 37°C for 15 minutes. Treat other samples in the same way, but stagger at 10 minute intervals.

4. Radioactive Labelling

Add L-5-^{3}H-proline (0.5 ml of 100 µCi/ml; 3.7 MBq/ml stock in MEM) to tubes 120. Add a further 25 µl ascorbate/BAPN. Incubate tendons at 37°C for 30 minutes.

5. Chase

Centrifuge tubes 120 at 11,600 g for 30 seconds. Remove supernatant, add 1 ml 1mg/ml unlabelled proline in MEM to tissues, whirlimix briefly and recentrifuge. Remove supernatant, and add 0.75 ml of 0.1 mg/ml proline in MEM. Add cycloheximide (0.25 ml of 1 mg/ml stock in MEM). Incubate tubes at 37°C for appropriate chase time (0, 10, 30, 60, 90, 120 minutes).

6. Extraction

Stop the incubation at appropriate time with 1 ml 1 M Tris-HCl/250 mM EDTA/100 mM NEM/11 mM PMSF pH 7.4 (20°C). Vortex tubes, spin down tissues as above. Remove supernatant, and add 1 ml of 1 M NaCl/50 mM Tris-HCl/25 mM EDTA / 10 mM NEM/0.9 mM PMSF, pH 7.4 (20°C). Extract proteins by shaking overnight at 4°C. Spin down the tissues, dialyze supernatants against 0.15 M NaCl/50 mM Tris-HCl/25 mM EDTA / 10 mM NEM/ 1 mM PMSF, pH 7.4 (20°C) for 4 hours.

7. SDS-PAGE

Determine number of counts in samples by scintillation counting of 10 µl aliquots. Load samples (equal number of counts in each track) on 6% acrylamide gels and carry out SDS-PAGE. Analyze by fluorography and densitometry.

Table 3.1 Prototype pulse-chase protocol devised in order to obtain values for the rate constants of procollagen processing.

accordance with previous estimates of the secretion time in tendon (Harwood, Grant and Jackson, 1976; Kao, Mai and Chou, 1982). After a pulse time of 20 minutes and examination of the fluorograms obtained from experiments using this revised protocol, it could be seen that the labelled procollagen at the end of the pulse had not been processed. Hence, in the final protocol (Table 3.2) a pulse-labelling period of 20 minutes was used. The initial amount of radioactivity used in the pulse (50 μCi/ml; 1.85 MBq/ml) was reduced to 25 μCi/ml (0.925 MBq/ml) as this resulted in a sufficiently high specific activity for detection of the radioactive proteins, and also it was more economical.

3.4.2 Length of the Chase Time

In previous pulse-chase studies on embryonic chick tendons, processing was complete by approximately 40 minutes after the administration of label (Leung et al, 1979) or 195 minutes after administration of label (Uitto and Lichtenstein, 1976). Initially, the tissues were chased for up to 120 minutes, which together with the pulse time meant that experiments were carried out for 150 minutes after the administration of label. However, after the pulse was reduced to 20 minutes, samples were also taken at 180 minutes of chase time (200 minutes after the administration of label).

At first, samples were taken at 0, 10, 30, 60, 90 and 120 minutes of chase time. However, for greater accuracy in fitting curves to the data, more chase times were used (Atkins, 1969). More chase time points were selected between 0 and 10 minutes, as in this period changes were occurring in the concentrations of the intermediates. Thus, the range of chase times was extended with samples being taken at 0, 4, 6, 8, 10, 15, 20, 30, 60, 90, 120, and 180 minutes.

Table 3.2 The final pulse-chase protocol. Except where specified below, all procedures and materials were the same as in Table 3.1.

---

1. Dissection

Semi-sterile procedures used throughout. Dissect tendons in HBSS/1% PS. Measure length of third toe.

2. Preincubation

Place tendons in Eppendorf tube (duplicate samples) containing 0.45 ml MEM + 25 µl ascorbate/BAPN stock and incubate for 15 minutes at 37°C.

3. Pulse

Add 0.5 ml L-5-^{3}H proline (from 50 µCi/ml stock) and incubate for 15 minutes.

4. Chase

Centrifugation at 11,600 g for 30 seconds. Remove supernatant, add 1 ml 1mg/ml proline, vortex briefly, recentrifuge as above. Remove supernatant, add 0.95 ml 0.1 mg/ml proline + 50 µl ascorbate/BAPN stock. Incubate tubes for the appropriate chase time: 0, 4, 6, 8, 10, 15, 20, 30, 60, 90, 120 and 180 minutes. Stop incubation by adding 1/10th the volume of 1 M Tris-HCl/250 mM EDTA/100 mM NEM/ 11 mM PMSF pH 7.4. Vortex thoroughly, then centrifuge at 11,600 g for 30 seconds, remove the supernatant and add 250 µl of 1 M NaCl/50 mM Tris-HCl/25 mM EDTA/10 mM NEM/0.9 mM PMSF pH 7.4.

5. Extraction

Extract at 4°C with shaking for 40 hours. Centrifuge tissues at 11,600 g. Count 10 µl aliquots by scintillation counting, and load appropriate volumes of samples on SDS-PAGE. Analyse data by fluorography and densitometry.

3.4.3 Use of cycloheximide

In the initial protocol (Table 3.1) cycloheximide (250 μg/ml) was included in the medium, in order to prevent further incorporation of radioactive label into protein synthesized during the chase period. Cycloheximide acts by binding to the peptidyl transferase activity of the 60S subunit of the ribosome, thus preventing any further elongation (Stryer, 1981). Cycloheximide was found to be an effective inhibitor of further incorporation of the radioactive label into protein (Fessler, Morris and Fessler, 1975; Morris et al, 1975; Uitto and Lichtenstein, 1976; Uitto, 1977; Leung et al, 1979; Limeback and Sodek, 1979; Uitto, Allan and Polak, 1979; Fessler, Timpl and Fessler, 1981; Ryhanen et al, 1982). However, for kinetic studies the use of cycloheximide has drawbacks. The 'chase' period follows the processing of the ^{3}H-labelled procollagen by the processing enzymes. As the 'chase' period proceeds, the amount of ^{3}H-substrate available for enzymatic processing decreases, which will affect the rate of cleavage by the enzymes if there is no further protein synthesized to replenish the pool of procollagen. This follows from the basic statement of enzyme kinetics,

$$v = k[E][S]$$

i.e. the rate of an enzyme reaction depends on the substrate concentration. Thus, the rate of processing observed in the presence of cycloheximide may differ from the rate observed in the absence of cycloheximide. Hence the use of cycloheximide in further experiments was abolished.

It was necessary to stop the incorporation of ^{3}H-proline present during the pulse into procollagen during the chase. The method chosen was replacement of the radioactive medium with unlabelled proline in MEM (Bateman et al, 1986). The protocol was revised as follows:

a) at the end of the pulse, the tissues were centrifuged (MSE, MicroCentaur, 11,600 g).

b) the radioactive medium was removed and 1 ml of 1 mg/ml unlabelled proline solution in MEM was added to prevent further incorporation of the label into protein.

c) tissues were vortexed briefly and recentrifuged;

d) the medium was removed and replaced with 0.1 mg/ml unlabelled proline in MEM (Fessler, Timpl and Fessler, 1981).

L-proline at a concentration of 10 mM (i.e. about 1 mg/ml) was effective in inhibiting further incorporation of radioactive label (Bateman et al, 1986). This concentration of unlabelled proline was used to wash the tissues and remove the label. A lower concentration of proline was used throughout the pulse period, as it was thought that using 10 mM L-proline might affect procollagen synthesis due to the large size of the proline pool. The specific activity of the L-5-^{3}H-proline was 30 Ci/mmol. Hence, incubating with 0.1 mg/ml unlabelled proline constitutes a 1000-fold excess. This should be enough to stop incorporation of any L-5-^{3}H-proline remaining after the wash.

3.4.4 Extraction of the tissues

Classical methods for the extraction of procollagens and collagens involve salt solutions at neutral pH (0.15 to 2M NaCl), or 0.5 M acetic acid (Miller and Gay, 1982; Miller and Rhodes, 1982). At low temperatures these agents are capable of dissociating fibrillar collagens into largely monomeric molecules. Neutral salt solutions preferentially extract newly synthesized molecules which have not yet been covalently cross-linked (Miller and Gay, 1982). Dilute acid induces swelling of the fibres and promotes the dissociation of intermolecular cross-links containing aldimine bonds (Section 1.6; Fitch et al, 1988), though intermolecular cross-links containing the keto-imine

cross-links are less labile. Pepsin in 0.5 M acetic acid has also been used to dissolve fibres in cases where the other two extraction methods result in low yields. The use of pepsin results in the release of small non-collagenous sequences and serves to destabilize the fibril resulting in release of the molecule (Miller and Gay, 1982). However, this method was not suitable for the present study, as pepsin cleaves in the non-helical regions of the molecule resulting either in shortened or completely removed globular extensions. As the object of this work was to examine the enzymatic processing of these regions by procollagen C-proteinase and procollagen N-proteinase, pepsin could not be used.

For the purposes of the pulse-chase protocol, a neutral salt solution was considered to be the most appropriate method of extraction of molecules containing collagenous sequences. It was decided not to use 0.5 M acetic acid for extraction, as the low pH of the acid might result in proteolytic activity of cathepsins.

A concentration of 1 M NaCl was used, as the efficiency of extraction is increased at higher salt concentrations (Miller and Rhodes, 1982). A neutral salt solution will also extract other collagen types as well as type I collagen (Byers et al, 1974; Timpl et al, 1975). Tris buffer (50 mM) was also present (Miller and Rhodes, 1982). Protease inhibitors (EDTA, 25 mM; NEM, 10 mM; and PMSF, 0.9 mM) were present in order to minimize proteolytic cleavage of the protease-sensitive regions of the molecules.

Initially, tissues were extracted with 1 ml of 1 M NaCl/ 0.05 M Tris-HCl/ 25 mM EDTA/ 10 mM NEM/ 0.9 M PMSF pH 7.4, followed by dialysis against a solution containing 0.15 M NaCl or precipitation with ammonium sulphate (final concentration 17.6%). However for convenience a third protocol was devised. After stopping the chase with inhibitor stock solution, this was removed by centrifugation and 250 μl of the 1 M salt solution

containing inhibitors was added. The tissues were extracted by shaking for 40 hours at 4°C. The intact tissues were then centrifuged and the supernatants were loaded directly onto SDS-PAGE.

In a limited number of experiments 50 mM Tris-HCl/2.5 mM EDTA/2% SDS pH 7.4 was used as an extracting agent. Examination of the collagenous components on SDS-PAGE indicated that greater amounts of procollagens were present throughout the chase even at chase times of 90 minutes (Figure 3.2). It is not clear why the results from the SDS extraction and the neutral salt extraction procedures should differ. One possibility is that the SDS was extracting intracellular ^{3}H-labelled procollagen which was present in a second intracellular pool which has been observed to have a half time of secretion of about 100 minutes (Kao, Berg and Prockop, 1977; Kao, Prockop and Berg, 1979).

3.5 Assumptions of the protocol

It is assumed that a) incorporation of ^{3}H- proline ceases after the pulse; b) type I procollagens, pC- and pN-collagens are extracted to the same degree by the 1 M salt solution, with no preferential extraction of one of the species over another; c) radiolabelled proline behaves as a perfect tracer (Atkins, 1969) such that the radiolabelled proteins are processed to the same degree and at the same rate as unlabelled ones.

3.6 Model Fitting

The pulse-chase data were used to obtain values for the rate constants k_1, k_2, k_3 and k_4.

If during the time course of an experiment the concentrations of all the components and the rates of all the reactions are constant, then the system is in a steady state. If a tracer molecule of substance A is introduced

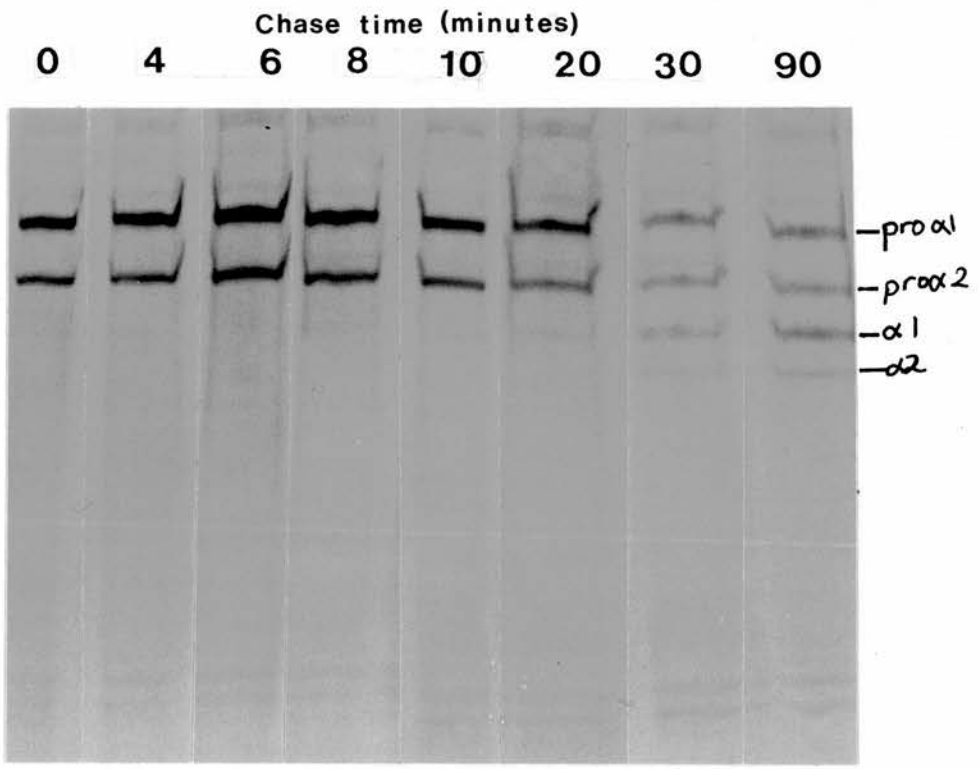

Figure 3.2 Fluorogram showing the time course of processing of type I procollagen. Experimental details were as described in Table 3.2, except that 50 mM Tris-HCl/2.5 mM EDTA/2% SDS pH 7.4 was used to extract the tendons. Note the persistence of proα chains at later chase time points compared with tendons extracted in 1 M NaCl (e.g. Figure 4.2).

into the system, its disappearance depends only on its concentration, and hence the removal of the tracer can be described by a single exponential curve.

$$\text{rate of disappearance} = k_1[a]$$

where [a] = the amount of A

k_1 = first order rate constant

i.e. the rate can be described by an ordinary linear differential equation. The substance A can be described as a "compartment", which is defined as the quantity of a substance which has uniform and distinguishable kinetics of transport and transformation (Atkins, 1969). Hence, procollagen, pC-collagen, pN-collagen and collagen can be denoted A, B, C and D respectively. If a small amount of ^{3}H-procollagen is introduced into the system at t=0, then the kinetics of the tracer can be described by four rate constants k_1, k_2, k_3 and k_4, representing the reactions pro to pC, pro to pN, pC to collagen and pN to collagen (Figure 3.1) in a set of ordinary linear differential equations

$$\frac{d[a]}{dt} = -(k_1 + k_2)[a] \qquad (1)$$

$$\frac{d[b]}{dt} = k_1[a] - k_3[b] \qquad (2)$$

$$\frac{d[c]}{dt} = k_2[a] - k_4[c] \qquad (3)$$

$$\frac{d[d]}{dt} = k_3[b] + k_4[c] \qquad (4)$$

Upper case letters denote the name of a substance in the compartment; lower case letters denote the concentration of the substance in the compartment (Figure 3.3).

These equations can be solved by a mathematical procedure (Laplace transform) in which the concentration of the tracer at any time is represented as a function of the initial concentration, the rate constant and the time. The equations were solved by Dr G L Atkins and their solution is given in Figure 3.4.

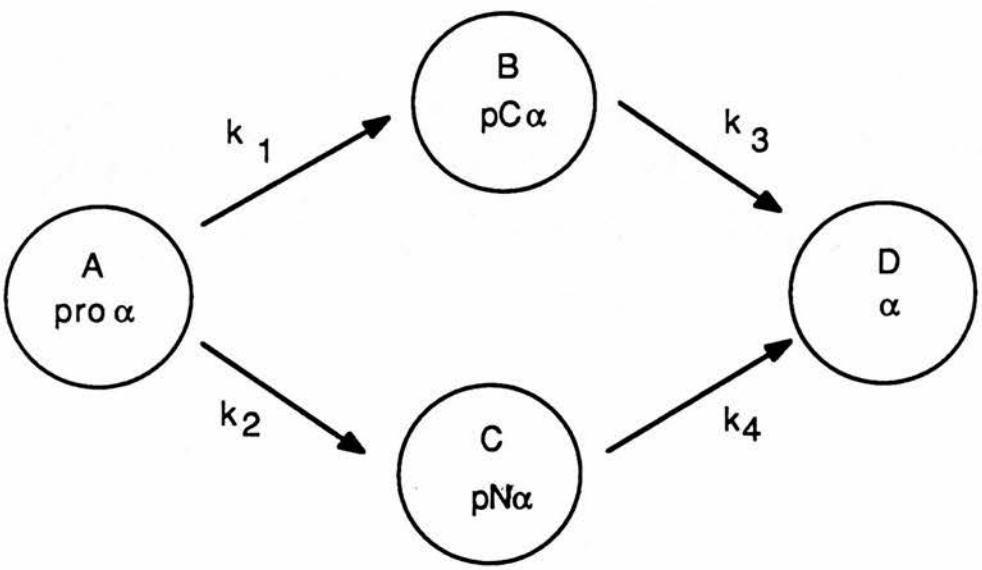

Figure 3.3 Scheme of procollagen processing showing the rate constants of the steps of processing and depicting each of the four species as a compartment (procollagen = A, pC-collagen = B, pN-collagen = C, collagen = D).

Procollagen processing can be represented by three compartments; for proα chains (A) the tracer goes through one compartment represented by a single exponential (Figure 3.4, equation 5). For pC- and pN-collagen the tracer goes through two compartments (enters from the proα compartment and leaves to the α compartment), and the equation solution is a sum of two exponentials (Equations 6 and 7). The tracer procollagen goes through three compartments in order to be transformed into α chains and thus equation 8 describing the concentration of D (α chains) is a sum of three exponential terms (for a fuller discussion, see Atkins, 1969).

3.6.1 Data

The data were obtained by introducing a known amount of L-5-^{3}H-proline (25 μCi/ml; 0.925 MBq/ml), taking samples at subsequent time intervals, determining the amount of radioactivity in each compartment and analyzing the data in order to calculate the rate constants k_1, k_2, k_3 and k_4.

Data points were collected as described (Section 3.8). Gel tracks on the fluorogram were scanned using the Chromoscan 3 (Joyce Loebl) and the amount of radioactivity in each band was determined as a percentage of the total radioactivity in the collagenous bands in each gel track. Thus, for each experiment a data set was obtained describing the change in percentage of each of the bands present as a function of time: proα1, proα2, pCα1, pCα2, pNα1, pNα2, α1 and α2. Data were collected for each component at each developmental stage studied.

For each data point a 'weighting factor' was ascribed. This weighting factor was the inverse of the variance (Wilkinson, 1961; Askelöf, Korsfeldt and Mannervik, 1976). For the purposes of curve fitting it is necessary to assume that the error structure of each data point has a normal (Gaussian) distribution, and this is probably the

$$a = a(o) \cdot e^{-(k_1 + k_2)t} \tag{5}$$

$$b = \frac{k_1 . a(o)}{(k_3 - k_1 - k_2)} \left[e^{-(k_1 + k_2)t} - e^{-k_3 t} \right] \tag{6}$$

$$c = \frac{k_2 . a(o)}{(k_4 - k_1 - k_2)} \left[e^{-(k_1 + k_2)t} - e^{-k_4 t} \right] \tag{7}$$

$$d = a(o)\left[1 - \frac{(k_3 . k_4 - k_2 . k_4 - k_1 . k_3)}{(k_1 + k_2 - k_3)(k_1 + k_2 - k_4)} e^{-(k_1 + k_2)t} - \frac{k_1}{(k_1 + k_2 - k_3)} e^{-k_3 t} - \frac{k_2}{(k_1 + k_2 - k_4)} e^{-k_4 t}\right] \tag{8}$$

Figure 3.4 Solution of the linear differential equations 1-4 (see text) describing the kinetics of ^{3}H-labelled procollagen (procollagen = A, pC-collagen = B, pN-collagen = C, collagen = D).

case (Atkins, 1969; Askelöf, Korsfeldt and Mannervik, 1976). The variance could not be determined for cases where only one data point was available, and in such situations the data point was given an arbitrary value of 0.1 for the weight.

3.6.2 Model Fitting

Curve fitting is optimized when the sum of the squares of the residuals is a minimum (Figure 3.5), i.e.

$$\Sigma wf.residuals^2$$

is a minimum, where wf = weighting factor. The values of the constants were adjusted until this condition was met.

An algorithm was used in the minimization program in which initial estimates of the rate constants were optimized by a gradient method (Marquardt, 1963; Cleland, 1967). For a given data set, a contour joins all the possible combinations of parameters which give the same values of sums of squares of the residuals (contours of equal variance). A hypothetical set of contours is shown in Figure 3.6. If parameters k_1 and k_2 are being compared, then Figure 3.6 is two-dimensional. In the current system there were four parameters to be determined, and thus the contour diagram is four-dimensional. For the purpose of simplicity, let us suppose that the system is two dimensional, with two parameters to be determined, k_1 and k_2. Then, let the initial estimate be represented on Figure 3.6 as x. The program calculates the direction of the steepest gradient until a trough is found (y in the diagram). The program then uses y as the next estimate and sets off again until a new minimum is found. The iteration continues until the minimum point is obtained (m in Figure 3.6). At this point the sum of the squares of the residuals of the parameters will be a minimum. In the current system, four parameters were determined simultaneously.

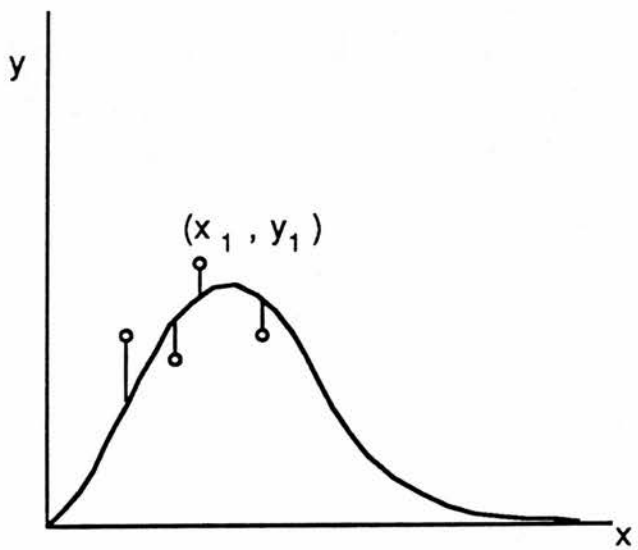

Figure 3.5 Diagram showing a hypothetical fitted curve in relation to hypothetical experimental data. At an experimental point of (x,y), the quantity $(y_i - y)$ is the residual. Thus, the residual, or the sum of the squares of the residuals, is a measure of how well the curve fits the experimental data points.

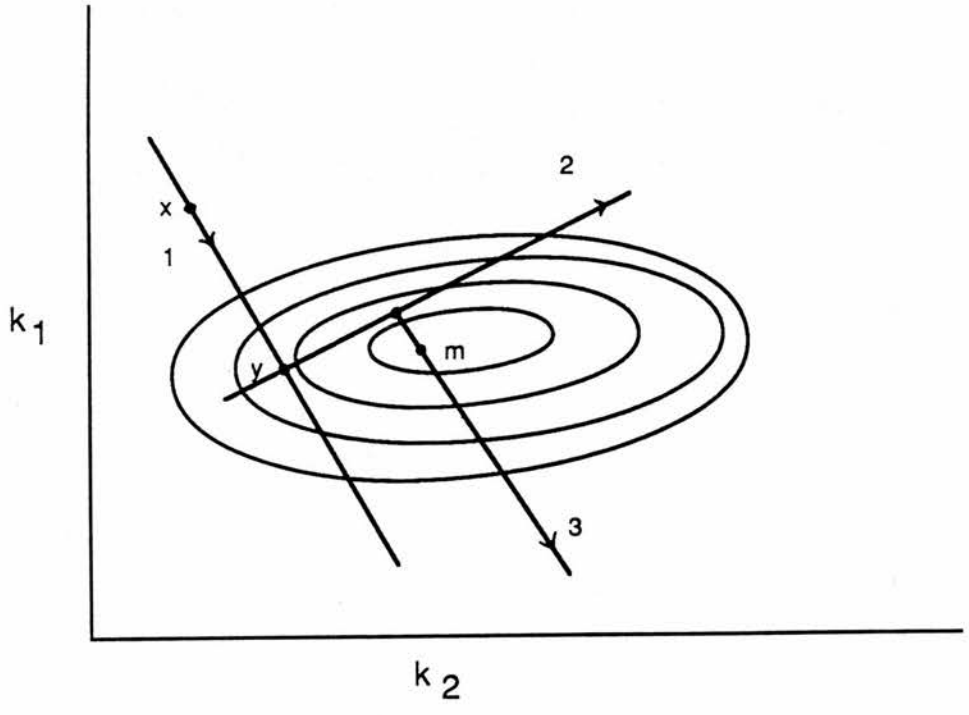

Figure 3.6 A hypothetical contour map used to show the gradient method of curve fitting.

A contour joins all the possible combinations of parameters which give the same values of the sums of squares of the residuals, i.e. they are contours of equal variance. An initial estimate, x, is programmed into the computer. An algorithm is then used to calculate the line of steepest gradient until a trough is found (the point y). y is used as the new initial estimate and the program sets off again until another trough is found, and so on, until the min SSQ is located (m).

3.6.3 Assumptions and Limitations of the Analysis

In order that the data obtained are an accurate description of the system, several assumptions were made;

a) The ^{3}H-labelled molecule acts as a perfect tracer. This means that the system should be unable to distinguish between the radioactively labelled molecules and the untritiated ones, and that both are utilized in exactly the same way. There should be no exchange of the isotope between the labelled procollagen and other compounds, and the tracer should not affect the steady state of the system (Atkins, 1969).

b) The system is assumed to be in a steady state, i.e. the turnover rate between compartments is constant and the amount of the substances in each compartment remain constant (Atkins, 1969).

c) There is no distinction in the participation of old and new molecules in the processes in the compartments (Atkins, 1969).

d) Within the compartment instantaneous and homogeneous mixing of the molecules occurs, or the time of mixing is very small compared with the turnover time of the substance (Atkins, 1969).

These data were not obtained by using ^{3}H-procollagen as a tracer, but by introducing ^{3}H-proline into the system and allowing time for the ^{3}H-pro to be incorporated into the prolyl-tRNA pool, and thence for the procollagen to be synthesized, transported through the cell and secreted. A complete description of the system would require that the model should include compartments for the processes leading to secretion, as well as for the processes after secretion. However, this would require more data to be collected to determine i) the amount of ^{3}H-pro coupled to tRNA; ii) the rate of incorporation of ^{3}H-prolyl tRNA into procollagen; iii) the rates of transport between intracellular organelles; iv) the rate of secretion. The present experiments do not allow data for i), ii), iii)

and iv) to be determined. Even if such data were available, it might not be possible to obtain values for the parameters involved due to a lack of detailed knowledge of the systems i), ii), iii) and iv). Technical difficulties might result in poor data for which parameters could not be obtained. Thus, the best approach is to start off with the simpler model of processing of procollagen. Hence, although the analysis is limited, it is justified on the grounds that it is easier to generate good data from a simple system, and that it might not be possible to obtain values for the rate constants from a more comprehensive model if unknown factors operate. Also, the object of the analysis was to compare processing in tendon and cornea at different developmental stages. If the processes leading up to and including secretion have comparable kinetics in the two tissues then the comparison is valid.

3.6.4 Validity of the Curve Fitting Technique

After the model fitting has been carried out, it is necessary to check that the model is an appropriate description of the system of procollagen processing, by testing for "goodness of fit". Various criteria were used:

a) Standard deviation (SD) of the parameters. If the standard deviation of the constant is less than 10%, then the fit can be described as good; if the SD is between 10-20% then the fit is less good and if the SDs exceed 25% of the values then another model must be sought to describe the data (Cleland, 1967).

b) Residual plots. Another way of testing for goodness of fit is to construct residual plots. The value of the residual for each chase time point is assigned a positive or negative value depending on whether the residual is above (positive) or below (negative) the fitted curve. The ratio of positive to negative residuals should be about one, i.e. a good fit should result in equal numbers of

positive and negative residuals (Askelöf, Korsfeldt and Mannervik, 1976). This can be confirmed by carrying out runs tests, i.e. the values of the residuals are examined to see how many times the sign (positive or negative) changes along the residual plot. The more often the sign changes, the better the fit. The runs test can indicate whether the fit is better in one part of the fitted curve than another-a bad fit may be present if there are clusters of the same sign along the fitted curve.

3.7 Efficiency of extraction

It was important to determine the efficiency of extraction of the radioactive proteins with the 1 M salt solution. Ideally, most of the ^{3}H-proline labelled protein would be extracted by this procedure. It was decided to estimate the efficiency of extraction of the radiolabelled proteins simply by comparing the amounts of ^{3}H-hypro (i.e., ^{3}H-pro which had become incorporated into collagenous proteins during the period of the pulse) in the extracts to the amounts remaining in the tissues after extraction.

It was necessary to use a method which gave quick and easy separation of ^{3}H-hypro from the ^{3}H-pro. A method was supplied by Dr R McAnulty (personal communication), details of which have been published (Turner et al, 1986; McAnulty and Laurent, 1987). The method of McAnulty was simplified and changed to fit the needs of the assay.

3.7.1 Preparation of Samples

Samples at 0, 30 and 180 minutes of chase time were assayed for efficiency of extraction. The 1 M salt extract was removed from the tissues with a syringe and transferred to an Eppendorf tube, in order to remove as much of the extract volume as possible from the tissues. The volume of the extracted material was measured. Water

(0.5 ml) was then added to the Eppendorf tube containing the tissue. The tube was then Whirlimixed (Fisons) to wash the tissue, and then recentrifuged. The wash was added to the tube containing the extracted material. This washing step was repeated twice. The tissues were then transferred to a ground glass homogenizer, and homogenized in 1 ml 1.5 M HCl. Tissues from 17-day embryos were tougher and before homogenization were transferred to a glass Petri dish and chopped with a scalpel before transfer to the homogenizer. This resulted in small losses of material due to the transfer process.

The homogenates and extracts were then transferred to glass test tubes which had been narrowed at the neck. Concentrated HCl was added to the samples to produce a final concentration of 6 M; the tubes were sealed and the samples were hydrolyzed in an oven (Gallenkamp) at 116°C for 16 hours. After hydrolysis, the samples were applied to a column of Dowex AG50W-X8 which had been equilibrated with 1.5 M HCl.

3.7.2 Chromatography on Dowex columns

The resin used for the separation of the hypro and pro was Dowex AG50W-X8 (wet mesh size 200-400, i.e. particle size 106-250μ). This resin is a purified analytical grade cation exchange resin prepared from Dowex, consisting of styrene-divinylbenzene cross-linked copolymers in bead form derivatized with the strongly cationic sulphonic acid groups. The resin was supplied as H^+ ionic form. The H^+ ion is the exchangeable positive counterion associated with the negatively charged sulphonate groups.

Dowex AG50W-X8 is supplied as a dry powder. A slurry was formed by mixing with 1.5 M HCl. The volume of the slurry was about twice the final volume of the column (BioRad). At first a column of dimensions 2 x 15 cm was prepared. The slurry was poured into the column and packed and equilibrated with 1.5 M HCl. A peristaltic pump (Pharmacia

P1) was used to pump the 1.5 M HCl through the column, but it was found that the acid dissolved the tubing and also the plastic components of the delivery apparatus of the Pharmacia column. In order to circumvent this problem a glass column was constructed (2 x 17 cm) with no plastic components present, and the sample and acid used for elution were delivered by gravity. Placing the acid reservoir 20 cm above the top of the column gave a flow rate of 120 ml/hr. The fraction size was 2 ml, and this was constant throughout the period of elution.

Standard samples of ^{3}H-pro and ^{3}H-hypro (10,000 cpm) in 1.5 M HCl were applied separately to the column and the position of elution noted. A sample containing both radioactive amino acids (10,000 cpm of each) was also applied to the column (Figure 3.7). Recovery of counts off the column was greater than 90%. It can be seen that good separation of the amino acids was obtained under these conditions.

The mechanism of the separation is unclear. As both the amino acids are in 1.5 M HCl, they will both be fully positively charged at this pH, so the separation cannot be due to subtle differences in molecular charge. It is possible that molecular sieving effects contribute to the separation of the two amino acids. However, this is only speculative, and the mechanism of separation is still not known (McAnulty, personal communication).

The prepared samples were then applied to the Dowex columns by Mr I Purdom. For the separation of the ^{3}H-pro and ^{3}H-hypro, a sample volume of 2 ml was applied to the column:

a) Extract: 0.5 ml hydrolyzed sample + 1.5 ml water

b) Homogenized tissue: after hydrolysis the homogenate was centrifuged at 11,600 g (MSE MicroCentaur) for 5 minutes to precipitate out any undissolved material. A sample volume of 0.5 ml + 1.5 ml water was loaded onto the column. The pellet from b) was tested for the presence of radioactivity by scintillation counting.

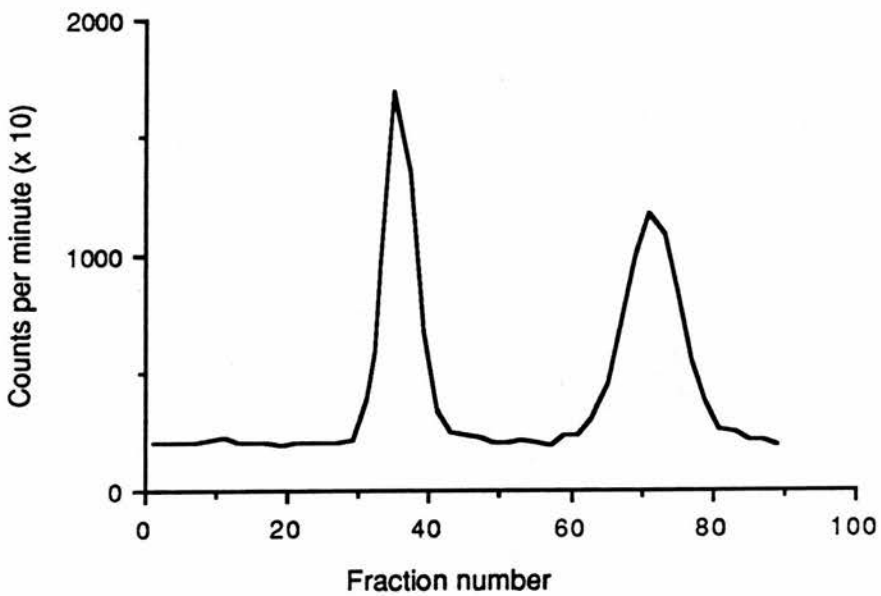

Figure 3.7 Elution profile showing the separation of a mixture of ^{3}H-pro and ^{3}H-hypro (10,000 cpm of each) on a column of Dowex AG50W-X8. The fraction volume was 2 ml and the flow rate was 120 ml/hr. The peak eluting 60-80 ml of 1.5 M HCl had been applied is hypro; the peak of radioactivity at 130-160 ml is proline.

For analysis of the fractions, samples of 200 μl were taken, and 200 μl water was added. Due to the effects of HCl on chemiluminescence, the samples were left for at least one hour before liquid scintillation counting (Packard Tri-Carb Liquid Scintillation Spectrometer). The cpm in the sample loaded on the column were counted (10 μl sample + 390 μl water) so that recoveries of counts from the column could be estimated.

3.8 Calculation of Densitometric Data and Correction Factors

After pulse-chasing, SDS-PAGE and fluorography, the results for each chase time can be represented as bands on a gel track. Each band can be assigned to a species containing type I collagenous sequences on the basis of comparison with known locations (Prockop and Tuderman, 1982; Figure 3.8).

Thus, after scanning densitometry of a gel track, the values of the areas underneath the peaks were obtained. The area scanned is a slit 0.3 x 5 mm. As the width of the gel track can vary depending on the amount of protein present, this could result in inaccuracies due to underestimation of the value of the integral for bands with more protein present. The correction factor was:

Value of integral x (width of band in mm / 5)

Another correction was made for loss of ^{3}H-pro from a band resulting from removal of one or both of the propeptides. These correction factors are listed in Table 3.3 and are based on the adjustment necessary to compensate for loss of the propeptides. The correction factors were calculated from the sequence of chick type I procollagen (Galloway, 1982). The number of hypro + pro residues present in the various domains of the proα1(I) and proα2(I) chains were calculated. Data were combined from the protein and cDNA sequences. Where there were gaps in the chick type I sequence, data were taken from the

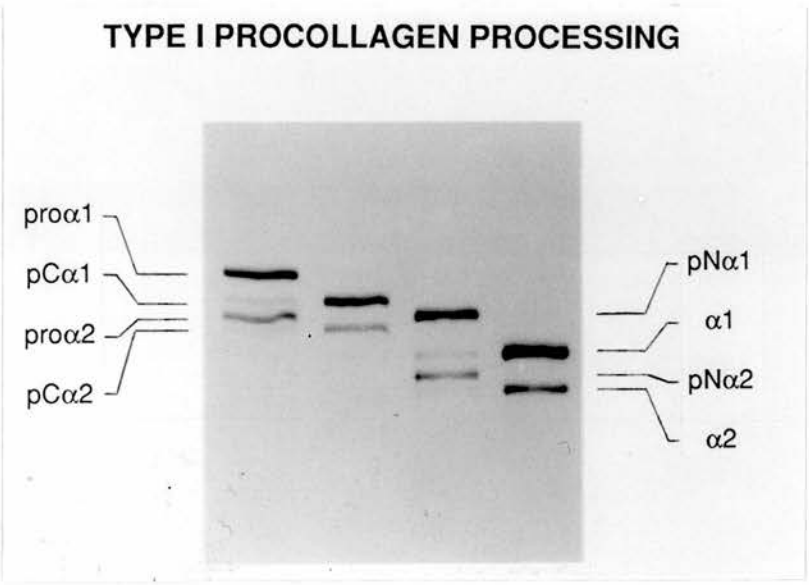

Figure 3.8 Tracks from a fluorogram showing the positions of migration of proα1, proα2, pCα1, pCα2, pNα1, pNα2, α1 and α2 chains on SDS-PAGE.

	Number of Pro + Hypro Residues in Domain	Correction Factor
Proα1(I) Chain		
C-Propeptide	10	0.963
N-Propeptide	25	0.907
Triple Helix and Telopeptides	235	0.87
	270	
Proα2(I) Chain		
C-Propeptide	12	0.953
N-Propeptide	19	0.926
Triple Helix and Telopeptides	225	0.879
	256	

Table 3.3 Table showing the correction factors used to correct the values of integrals obtained by densitometry of fluorograms of ^{3}H-proline labelled collagenous proteins in the pulse-chase experiments. The correction factors are necessary because there is ^{3}H-pro and ^{3}H-hypro in the propeptides and thus the value of the integrals of the pC, pN and αchains need to be corrected for the loss of ^{3}H. The number of pro and hypro residues were calculated from the data of Galloway (1982).

relevant regions of the bovine or rat sequences quoted (Galloway, 1982). Comparison of the bovine and rat sequences in these regions revealed a high degree of homology in the positions of pro and hypro, and thus it was assumed that these residues were conserved in the chick.

After the value of the integral for each band had been corrected for width and loss of ^{3}H, the integrals for a gel track were summed, and each corrected integral taken as a percentage relative to this total. Thus, the data do not refer to absolute protein concentrations, but to the relative amounts of each collagenous species present at each chase time point

No correction was made for the shape of the band, i.e. the shapes of the bands were assumed to be rectangular.

3.9 Electron Microscopy

At each stage of development studied, a sample of tissue was removed for electron microscopy to determine the mean fibril diameter. Tendons (3) or corneas (3) were washed in HBSS (containing 1% PS) and then placed in fixative (4% formaldehyde in 0.1 M sodium cacodylate).

The samples were processed for electron microscopy as described in Section 2.4.5 by Mrs C Cummings and Ms K Greenwood (University of Manchester).

3.9.1 Measurement of Fibril Diameters

Fibril diameters were measured using a Magiscan 2 (Joyce Loebl). The Magiscan 2 comprises a television camera linked to a computer. The negative of a section is illuminated with a light box and a selected field of view is transmitted to the screen via the television camera. The two points on the circumference of the fibril which represent the minimum width were delineated with a light pen and these were used to calculate the diameter of the

fibril using a program devised by Dr N Ward (University of Manchester). At least 450 measurements were recorded at each stage of development studied. A large number of measurements were taken because this gives a better distribution (Parry, Barnes and Craig, 1978). The micrographs were calibrated using a replica grating (2160 lines/mm).

CHAPTER 4

PROCOLLAGEN PROCESSING IN TENDON

4.1 Tendon

Tendons consist of fibrous tissue which provide a connection between muscle and muscle or muscle and bone, and transmit the tensile forces generated by muscle to these other elements (Kastelic, Galeski and Baer, 1978; Davison, 1982). Tendon structure is organized on a hierarchy of levels (Figure 4.1). Collagen molecules are packed into subfibrils of 10-20 nm in diameter. These subfibrils are then packed into fibrils 50-500nm in diameter. At the next level of organization, the fascicle or bundle, (80-320 μm), the fibrils are wrapped in a continuous loose connective tissue, the endotenon, which contains columns of fibroblasts. The tendon fibroblasts are elongated, with their long axes oriented parallel to the bundles of collagen, and long cytoplasmic processes. Fine vessels and nerves run to the inner region of the tendon from the endotenon. Finally, bundles of fascicles are wrapped within a sheath of loose and dense connective tissue, the paratenon (Kastelic, Galeski and Baer, 1978; Davison, 1982).

Under a polarizing microscope the fascicles possess a wavy zig-zag pattern known as "crimp". The crimps are registered, which means that all the "zigs" are oriented in the same direction and the "zags" are similarly registered around the circumference of the fibril (Kastelic, Galeski and Baer, 1978). The crimp may constitute a compliance mechanism in which a small length change in a tendon, arising from a straightening of the crimp, could prevent tendon damage during the initial stage of muscle contraction.

Relatively frictionless motion of the tendon is provided by the synovial cells, which secrete a buffer or lubricating fluid between the tendon and the sheath.

Figure 4.1 Diagram of the hierarchical organization of the collagen molecules (left) in a tendon (right). The triple helices of two molecules are indicated; the rest of the molecules are shown as single cylinders (not to scale). A view of quasi-hexagonal packing is shown (Section 1.6). Next, the banded fibril is shown; then the packed fibrils in the bundle are shown partially covered by a column of fibroblasts in the endotenon. Finally, the fibril bundles are enclosed by the tendon sheath. Adapted from Davison, 1982.

4.2 Development of the Tendon

Tendon development appears similar in rat (Greenlee and Ross, 1967), human (Chaplin and Greenlee, 1975) and the flexor digital tendon of the chicken (Greenlee, Beckham and Pike, 1974). Development of tendon in the chicken is relevant for this study and will be considered further.

Tendons derive from the mesenchyme. Mesenchymal cells differentiate into all the cell types in the tendon (tendon cells, synovial and interfascicular cells, elastin-producing cells of the vincula and cartilaginous cells of the tendon surface). At 4 days of development in the flexor digital tendon of the chick, mesenchymal cells are present in a loose stroma, but by 8 days of development condensations of cells have formed, with the cells showing the characteristic morphology of tendon fibroblasts. The tendon sheath is formed by condensation of cells around the tendon, and is fully formed by 12 days of development. A synovial cleft has formed betwen the tendon and its sheath, and vincula, which provide the vasculature for the tendon within the synovial sheath, are present. Fasciculation is obvious by day 13. By 17 days the synovial sheath cavity is completely formed and the volume of the tendon has increased. The specialized areas of the vincula and fibrocartilage are now well differentiated. At 21 days, the amount of collagen has increased, and by 22 days (post-hatching) a large increase in the amount and diameter of the collagen fibrils has occurred (Greenlee, Beckham and Pike, 1974). Development of the metatarsal tendon in the chick seems to follow a similar pattern. Mesenchymal cells differentiate into fibroblasts at 6-7 days (Fitton Jackson, 1956). At early stages in the metatarsal tendon, there are no clearly defined intercellular spaces. At 9-10 days intercellular spaces are more obvious, and by day 11, the condensations of cells begin to assume the appearance of bundles. Fasciculation is observed in day 14 embryos (McBride, Hahn

and Silver, 1985). No fasciculation is present at day 7. Day 10 embryos show early evidence of crimp formation development, as under polarized light birefringent fibres have an extinction pattern suggestive of crimp, i.e. the uniaxial orientation of fibrillar collagen is increasing, indicating that the tendon's ability to transmit tensile stress is developing. Subsequent development is characterized by the maturation of crimp and fascicle development and by an increased volume occupied by extracellular collagen (McBride, Hahn and Silver, 1985). Initially the fibril bundles are observed in close association to invaginated cell surfaces (days 7-10). By day 14 compartmentalization is distinct (McBride, Hahn and Silver, 1985). Bundles of 12-100 filaments are seen at 11 days of development (Fitton Jackson, 1956).

By day 11 in the chick, the fibrils have diameters of 12 nm (Fitton Jackson, 1956). Between 11 and 3 days the fibrils are 17 nm; 14 days, 25 nm; 16 days, 31 nm; day 20, 40 nm, and 75 nm by 6 months post-hatching. At 14 days the bundles are 0.5 μm in diameter, and by 6 months after hatching, the bundles are 3 μm in diameter. As the age of the embryo increases, the extracellular amounts of collagen increase, until in the adult tendon the cells are few (Fitton Jackson, 1956). The fibril packing fraction of the tendon increases with time, with greatest increases between 12 and 18 days of development (Fitton Jackson, 1956). As the collagen fibrils begin to dominate the matrix, the fibroblasts become arranged in columns. The greatest increase in the mass/unit length of the fibrils occurs between 12 and 18 days (Fitton Jackson, 1956). Hydroxyproline (mg/g dry weight) increases from 4.9 at 11 days, to 16 at 14 days, 43.5 at 17 days and 55.4 at 19 days (Scott and Hughes, 1986). The proportion of nucleic acids decreases with development, corresponding to the decrease in cellularity (Scott and Hughes, 1986).

Different values were obtained for diameters of chick tendons by Eikenberry and co-workers (1982). Electron

microscopy of embryonic chick tendons gave values increasing from 32 nm (11 day specimen) to 41 nm (17 day specimen) (Eikenberry et al, 1982). X-ray diffraction gave values ranging from 46 nm at 13 days to 58 nm at 19 days of development (Eikenberry, Brodsky and Parry, 1982). Diameter distributions of tendon fibril diameters for all species so far examined are unimodal in the embryo and at birth. The range of fibril diameters increases with age (Parry, Barnes and Craig, 1978; Parry, Craig and Barnes, 1978; Parry and Craig, 1984). Altricious animals have sharp distributions of diameters at birth, and these fibrils recommence growth after birth. Precocious mammals such as horse have broad distributions. Fibril diameters increase between birth and maturity and decrease between maturity and senescence. Most oriented tissues containing type I collagen which are subjected to long term stress have a bimodal distribution of diameters at maturity.

4.3 Collagen Types and Proteoglycans of Tendon

Matrix free tendon cells secrete procollagen which is considered to be most, if not all, type I (Uitto, Lichtenstein and Bauer, 1976). However, this is an artificial system and phenotypic changes may occur. Type VI collagen has been identified in chick tendon (Bruns et al, 1986), but the amount of type VI relative to the other types is not known. Type V collagen represents 8% of the acid-soluble collagen (Jimenez, Yankowski and Bashey, 1978). In contrast, a figure of 1% type V was quoted by Kao, Mai and Chou (1982). The α1'V chain is present in tendon matrix and appears to be present in trimers of $(\alpha 1')_3$ or as $(\alpha 1)(\alpha 1')(\alpha 2)$. The presence of type V in tendon is of interest in view of its copolymerization into fibrils with type I in the cornea (Birk et al, 1988; Section 1.6.4; 5.3). Screening of a cDNA tendon fibroblast library for collagen-like sequences revealed a clone which showed homologies to type IX collagen (Gordon, Gerecke and

Olsen, 1987). This was present in calvaria, sterna, tendon and 6 day embryonic cornea. Isolation of this type XII from 17 day chick embryo tendons showed that type XII represents 0.5% of the total tendon collagen (Dublet and van der Rest, 1987).

Keene et al (1987) detected uniform labelling of tendon fibrils with monoclonal antibodies against type III collagen. Using in vitro hybrid fibrils formed from mixtures of type I and type III collagen, it was shown that at least 1-2.5% of type III collagen must be present on the surface of tendon fibrils. This contrasts with chemical analysis of tendon collagen which indicates that no type III is present. The consequences for theories of fibril formation are not clear.

Glycosaminoglycans/proteoglycans constitute 0.03-0.3% of the wet weight of tendon (Parry and Craig, 1984). The amount of acidic polysaccharide decreased with development, as shown by the decrease in periodic acid and Hale stain intensity (Fitton Jackson, 1956 . HA decreased from 2.9 mg/g dry weight at 14 days to 0.8 mg/g by 19 days, and CS decreased from 3.8 mg/g to 3.4 mg/g (Scott and Hughes, 1986). CS was not present before 17 days of development. These data indicate a location for HA and CS outside the fibril, since because the total collagen/g tissue increases, the space available for HA or CS-PG decreases. DS appears to be present on the periphery of the collagen fibril (Scott, Orford and Hughes, 1981).

In adult tendon the small DS-PG constitutes more than 90% of the total PG (Vogel, Paulsson and Heinegård, 1984). Tendon also contains a large aggregating CS-PG (Poole, 1986, for references). The small DS-rich PG with a core protein of molecular weight 45,000 daltons has one DS chain per molecule, rich in iduronic acid. The DS-rich PG associates with rat tail tendon collagen at the d band (in the gap zone) (Figure 1.5; Scott and Orford, 1981). The small DS-PG of tendon affects fibrillogenesis in vitro (Vogel, Paulsson and Heinegård, 1984; Uldbjerg and

Danielsen, 1988; Section 1.6.4.1).

4.4 Diseases of Tendon

Tendons seem free from a disease which specifically affects this tissue. Each form of EDS and dermatosparaxis seems to show hyperextensibility of tendons, but these seem adequate for function. Tendon may be weakened by experimental or naturally occuring cases of lathyrism due to the presence of BAPN. Tendon weakening may also occur during treatment with penicillamine or cysteine which block aldehyde groups and prevent cross-linking (Davison, 1982). A diseased superficial flexor tendon from an 8.5 year old horse with 'contracted tendon' had a unimodal instead of a bimodal distribution of fibril diameters (Parry, Craig and Barnes, 1978). The number of elastic fibres present was reduced, and the D-period varied from 40-90 nm.

4.5 Previous Work on Processing

Matrix-free cells were isolated from the leg tendons of 17 day old chick embryos and incubated with ^{14}C-proline for 2 hours. The medium proteins were precipitated with 176 mg/ml ammonium sulphate and separated on DEAE-cellulose chromatography. A peak corresponding to the presence of pC-collagen was present (Uitto, Lichtenstein and Bauer, 1976). However, this was a long-term labelling study. Pulsing matrix-free cells from 17-day tendon resulted in conversion of ^{14}C-procollagen to pC-collagen (Uitto and Lichtenstein, 1976) and a pC intermediate was also seen in intact tendons (Leung *et al*, 1979).

The results obtained by the above workers were interpreted to indicate that procollagen processing in tendons occurs almost exclusively via pC-collagen, i.e. in terms of the present model, k_2 and k_4 are essentially zero (Figure 3.1). The work presented in this chapter was

carried out in order to determine all four rate constants for procollagen processing, k_1, k_2, k_3 and k_4.

4.6 Results

4.6.1 SDS-PAGE

After the pulse-chase experiment was completed and the proteins had been extracted from the tendons, the extracts were loaded on 6% acrylamide gels (Section 2.3.2). After SDS-PAGE, the gels were impregnated with PPO (Section 2.3.3), dried, and processed for fluorography (Section 2.3.3).

Gels of extracts from experiments at 12 and 17 days of embryonic development are shown (Figures 4.2 and 4.3 respectively). Two observations are apparent from examination of the gels. The first observation is that the 12 day type I procollagen appears to be processed exclusively via a pC-collagen intermediate. However, at later stages of development, increasing amounts of pN-collagen were visible on the films. The amount of pN-collagen seen was always low. This indicates that some change is occurring in the values of the rate constants as development proceeds.

4.6.2 Densitometry

The X-ray films were developed and the amounts of collagenous proteins determined using scanning densitometry (Section 2.3.5). Densitometric scans of chase time points t=0, 30 and 90 for 12 days and 17 days of development are shown in Figures 4.4 and 4.5. It can be seen from the gel scans that the peaks corresponding to the protein bands of the 12 day data were well resolved from each other. The peaks corresponding to the collagenous bands of the 17 day data were well resolved, except where pNα1 and proα2 could not be resolved from

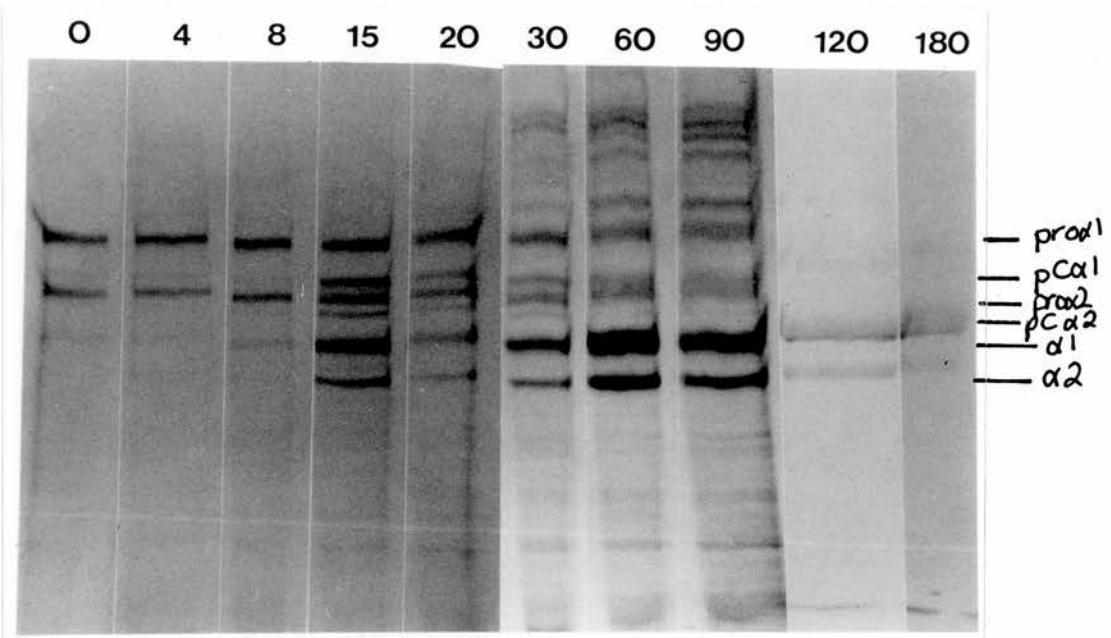

Figure 4.2 Fluorogram showing the time course of processing of type I procollagen in 12 day chick embryo tendons. Tendons were incubated with 25 μCi/ml L-5-^{3}H-proline for 20 minutes; after this time the medium containing the radioactive label was removed and replaced with 0.1 mg/ml unlabelled proline as described in Table 3.2. The tissues were extracted with a 1 M salt solution and the extracts were loaded on SDS-PAGE. The numbers above the gel tracks refer to the chase time in minutes.

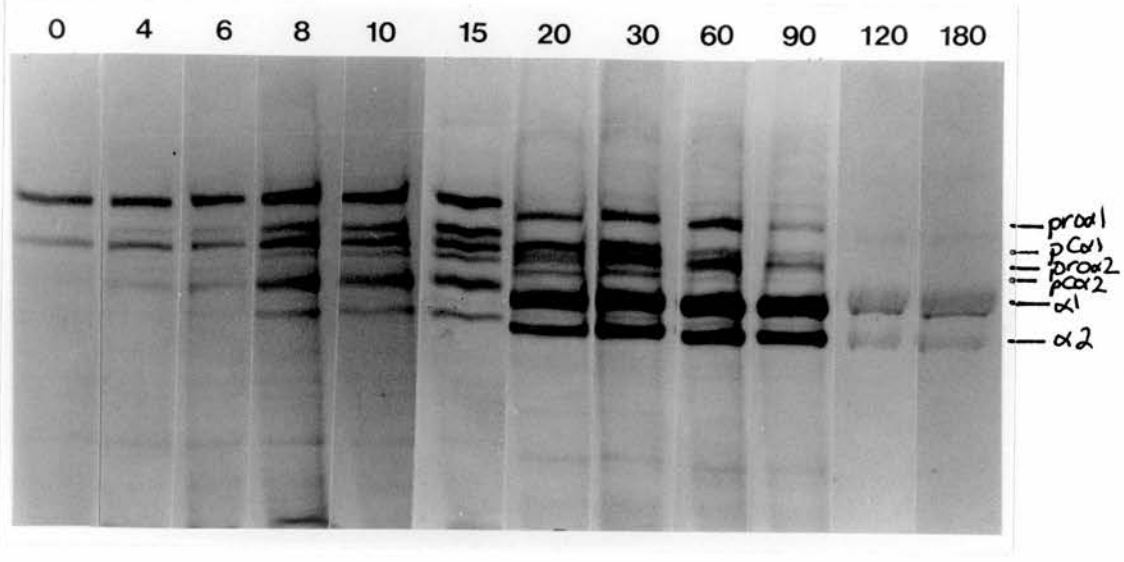

Figure 4.3 Fluorogram showing the time course of processing of type I procollagen in 17 day chick embryo tendons. Tendons were incubated with 25 μCi/ml L-5-^{3}H-proline for 20 minutes; after this time the medium containing the radioactive label was removed and replaced with 0.1 mg/ml unlabelled proline as described in Table 3.2. The tissues were extracted with a 1 M salt solution and the extracts were loaded on SDS-PAGE. The numbers above the gel tracks refer to the chase time in minutes.

each other. In this case, data for proα2/pNα1 was either omitted from the analysis, or a value of the integral for pNα1 was calculated as

$$[pro\alpha1] + [pC\alpha1] + [pN\alpha1] + [\alpha1] = 66.7\%$$
$$[pro\alpha2] + [pC\alpha2] + [pN\alpha2] + [\alpha2] = 33.3\%$$

Values of the integrals were corrected as described (Section 3.8). Data files were created for the α1-type chains; that is, the proα1, pCα1, pNα1 and α1 chains at each stage of development, and for the α2-type chains (proα2, pCα2, pNα2 and α2 chains) at each stage of development. The information in the data files was used to determine the four rate constants of processing k_1, k_2, k_3 and k_4 using the minimization program of Dr G Atkins (Section 3.6). Preliminary results were obtained by Dr Atkins, but later refined data were processed by myself using the minimization program.

4.6.3 Rate constants

It has been stated (Section 3.6) that the process of model fitting uses an algorithm to determine values for the parameters using a gradient method, i.e. the algorithm is reiterated until a minimum value of the weighted sums of squares of the residuals (min SSQ) is found. However, it is possible that the minimum point located by the program may not be a global minimum but only a local minimum. It is possible that there may be several local minima. If, for example, an initial estimate of a parameter of 0.05 is given, and then the initial estimates are varied by a factor of, say, +/- 0.02, Dr Atkins' program (the minimization program) might still locate the same minimum. If, however, an initial estimate is used which is a factor of 10 more or less than this, then another set of parameters corresponding to another minimum of the contour map may result. In summary, then, there may be more than one set of contours corresponding to minimum values of the sums of squares of residuals. The final

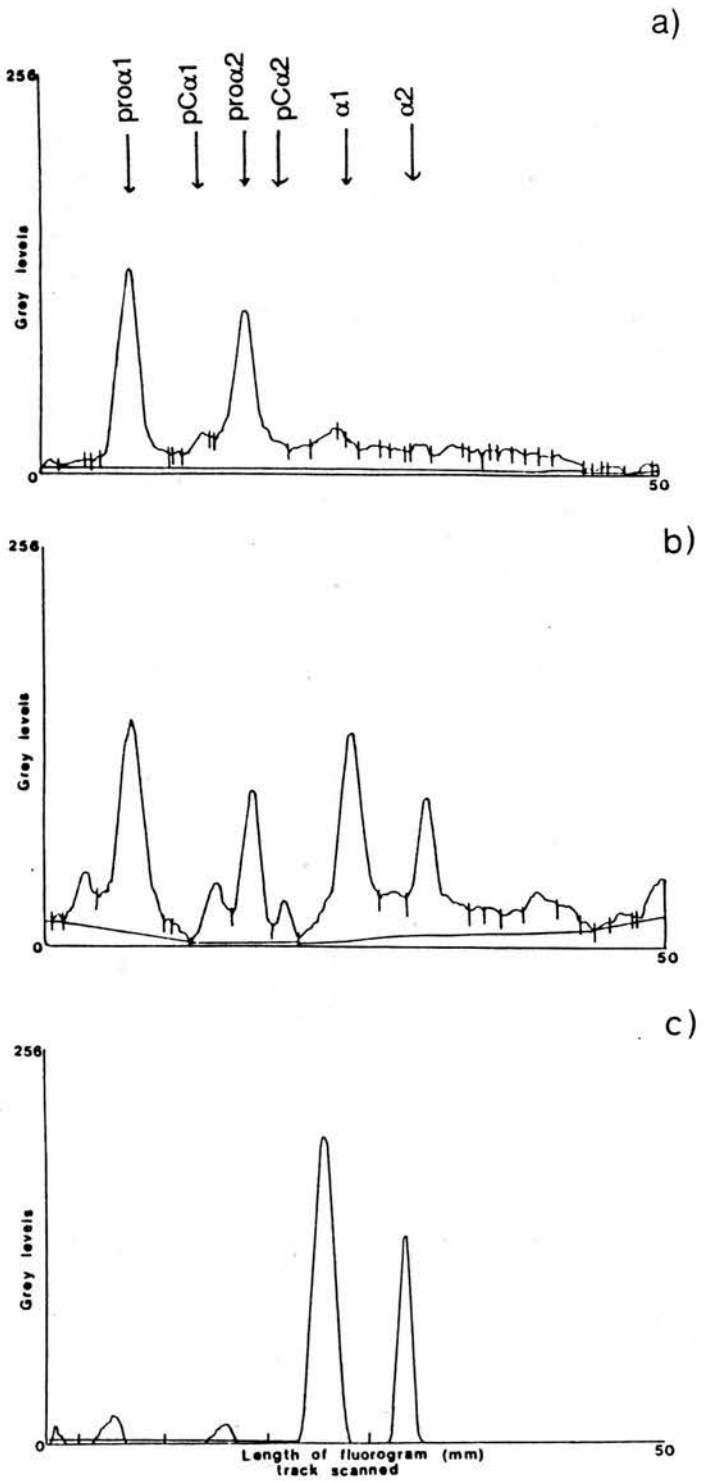

Figure 4.4 Densitometric scans of 12 day fluorogram for the chase time points 0, 30 and 90 minutes in the pulse-chase experiment. The fluorograms were scanned at 530 nm. Scan a), 0 minutes; b), 30 minutes; c), 90 minutes.

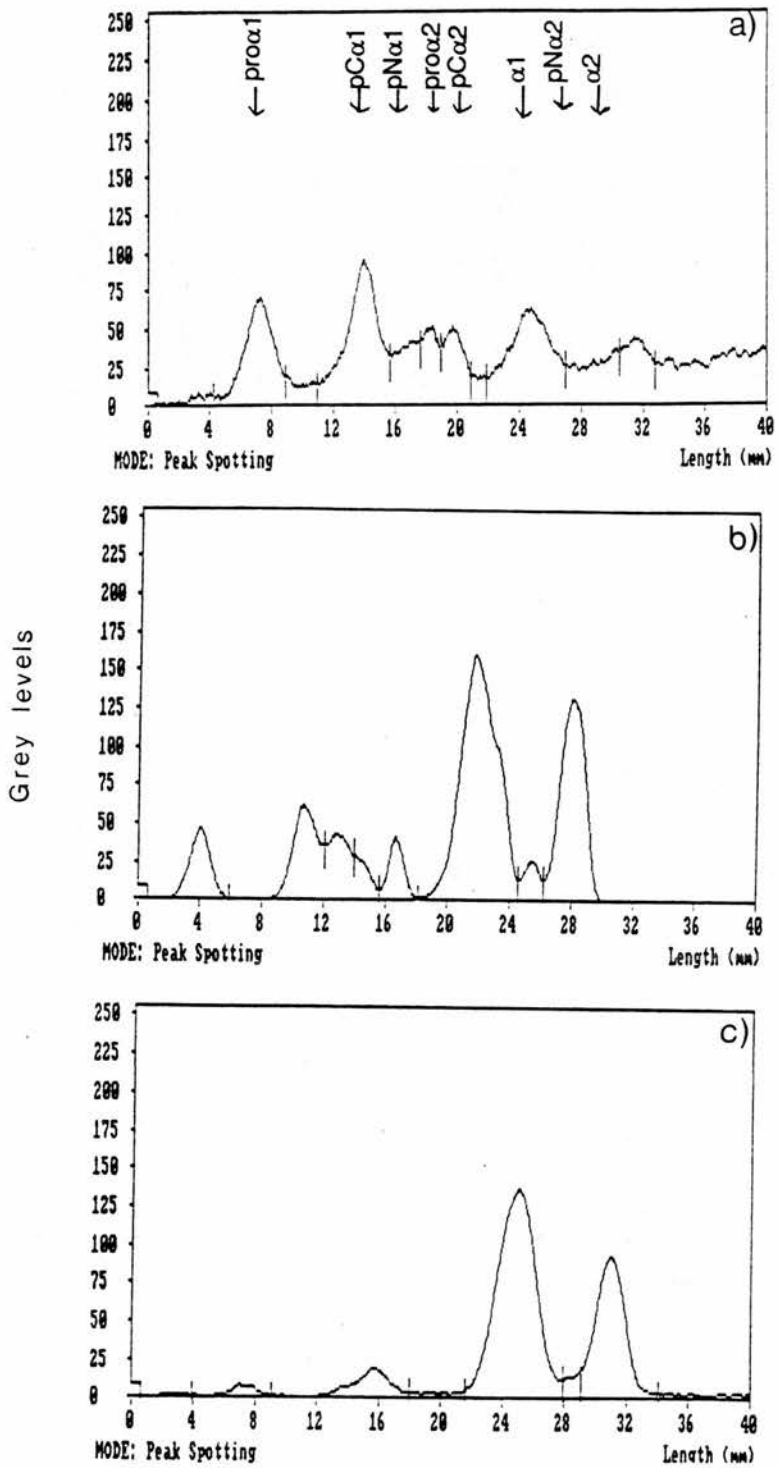

Figure 4.5 Densitometric scans of 17 day fluorogram for the chase time points 0, 30 and 90 minutes in the pulse-chase experiment. The fluorograms were scanned at 530 nm. Scan a), 0 minutes; b), 30 minutes; c), 90 minutes.

parameters obtained by the curve-fitting program may depend on the position of the initial estimates, so that initial estimates at a different "location" on the contour map may find a minimum point in another set of contours.

The possible existence of several local minima was tested systematically using a "global search" computer program devised by Dr D J S Hulmes, which calculated all the sums of squares of residuals over a 10,000 fold range for each of the rate constants from 0.0001 to 1 min^{-1}. Eleven cycles were carried out for each rate constant, with values increasing by a factor of 2.5 for each cycle. In this way approximate positions of local minima in the sums of squares of residuals (local troughs in the contour map) were located. The values of the rate constants at the local minima in the values of SSQ were then used as initial estimates for the minimization program, to see what final values of the rate constants resulted when approximate parameters for different local minima were used as initial estimates. The correct values for the parameters k_1, k_2, k_3 and k_4 were assumed to be those values for which the final sum of squares of residuals was a minimum. Not all of the local minima from the global search could be fitted by the minimization program. The minimization program occasionally failed, presumably because some values of the initial estimates of the rate constants resulted in denominators of zero (Figure 3.4). The results of fitting the local minima found by the global search program to the minimization program will be described for each curve in turn. The final values of the parameters are shown in Table 4.1.

12 day α1-type chains

Very little data could be obtained to determine the amounts of the pNα1 intermediate by densitometry of fluorograms. Consequently, the curves could not be fitted easily to the data for the twelve day α1-type chains, with only one local minimum being found. This could not produce fitted values of the parameters when used in the

α1-type chains

	12 days	14 days	17 days	19 days
k_1	0.025 +/- 0.00524	0.0305 +/- 0.00371	0.0468 +/- 0.0055	0.0837 +/- 0.00759
k_2	0.0082 +/- 0.0075	0.0534 +/- 0.0126	0.0527 +/- 0.00702	0.0087 +/- 0.0056
k_3	0.0566 +/- 0.0185	0.0269 +/- 0.00745	0.0358 +/- 0.00385	0.1202 +/- 0.0194
k_4	> 1	0.211 +/- 0.0739	0.236 +/- 0.04	0.000391 +/- 0.00479
min SSQ	619.8	159	138.5	252.9

α2-type chains

	12 days	14 days	17 days		19 days
k_1	0.0129 +/- 0.00254	0.0274 +/- 0.0049	0.0525 +/ 0.00975	0.0784 +/- 0.00963	0.0421 +/- 0.00611
k_2	0.0187 +/- 0.0036	0.0498 +/- 0.00876	0.0493 +/- 0.0119	0.0149 +/- 0.00392	0.0155 +/- 0.00292
k_3	0.0391 +/- 0.00642	0.0383 +/- 0.00775	0.0526 +/- 0.0127	0.108 +/- 0.0124	0.0751 +/- 0.0155
k_4	1.34 +/- 3	0.313 +/- 0.0789	0.204 + /- 0.061	0.017 +/- 0.011	0.0266 +/- 0.0101
min SSQ	16.6	55.2	113.5	112.7	4.28

Table 4.1 Values of the rate constants obtained by regression analysis of pulse-chase data for 12, 14, 17 and 19 day embryonic chick tendons. Two sets of values are shown for the 17 day α2-type chains, as these could not be distinguished from each other by the values of the minimum of the sums of squares of the residuals. Units: rate constant = k_i $minute^{-1}$ +/- standard deviation.

minimization program. Presumably the global search program did not include a set of values that could be fitted by the minimization program. Using a variety of initial estimates, the minimization program eventually calculated values for the parameters shown in Table 4.1, but because there was insufficient data available for the pNα1 intermediate, a value for k_4 could not be calculated.

12 day α2-type chains

Ten local minima were found for the 12 day α2-type chains using the global search program. Only one set of minima when used as initial estimates gave values of the rate constants (see Table 4.1). The value of k_4 must be treated with some caution as very little pNα2 was present.

14 day α1-type chains

In cases where the values of the parameters of the local minima located by the global search program were used as initial estimates and where fitting occurred, only one set of values for the rate constants were located, i.e. all values of initial estimates converged to the same global minimum (Table 4.1). The common feature of these initial estimates was that k_1 was less than or equal to k_2, which was less than k_4. If k_1 was equal to k_4, or if k_2 was equal to k_4, no fit was obtained.

14 day α2-type chains

Eight minima were located by the global search program. Four of these could not be fitted when the parameters were used as initial estimates in the minimization program. Three sets of estimates (low k_1 and k_2, high k_4) converged to give the values shown in Table 4.1, with a value of the min SSQ of 55.7. The fourth set of initial estimates had values of k_2 and k_4 less than k_1 and another minimum was found. The min SSQ for this set of parameters was 89.3. This latter set of rate constants was compatible with a model of processing almost exclusively via a pC-collagen intermediate (low k_2 and k_4) as opposed to the other parameters found (first cleavage random, k_4 large). Both sets of parameters are consistent with the visual

observations of the fluorogram. However, the possibility of processing exclusively via a pC-collagen intermediate was rejected because the value of the min SSQ was 89.3, higher than the other value of the min SSQ (55.7), and also because the standard deviation parameters of k_2 and k_4 were large (i.e. the fitted values were not reliable).

17 day α1-type chains

A similar pattern was found for the 17 day α1-type chains. A fit could be obtained with the values of the parameters from the local minima when both k_1 and k_2 were less than k_4. If k_1 or k_2 were of the same order of magnitude as k_4, then no fit was obtained. The value of the min SSQ was 138.5. Another trough was found when $k_1 = k_2 = 0.381$; $k_3 = 0.0244$ and $k_4 = 0.153$, resulting in fitted values similar to these initial estimates, with a min SSQ of 345. Hence, as the min SSQ was larger than the min SSQ of the values in Table 4.1, and also the standard deviation parameters were unacceptably high, these values were discarded.

17 day α2-type chains

A different situation was found for the α2-type chains. When k_4 was larger than k_1 and k_2, a pattern of rate constants similar to the rest of the data was obtained (Table 4.1). However, if k_2 and k_4 were less than or equal to k_1, then the alternative model was seen, with values of 0.0784, 0.0149, 0.108 and 0.017 for k_1, k_2, k_3 and k_4 respectively. The min SSQ of these two sets of constants were very similar. Hence, it is not possible to distinguish the two models from one another on the basis of the lowest value of the min SSQ.

19 day α1-type chains

The only values obtained from fitting the nineteen day type 1 initial estimates to the data resulted in parameters listed in Table 4.1, i.e. low k_2 and k_4, indicating an exclusive route of processing via pC-collagen. However, the standard deviations of k_2 and k_4 are large, indicating that the fit may not be good.

19 day α2-type chains

All the values obtained from fitting parameters from the local minima were of the same order of magnitude, i.e. k_4 had decreased 10-fold (Table 4.1). The value of the min SSQ was 4.28.

4.6.4 Validity of the curve fitting technique

4.6.4.1 Visual Inspection

The fitted curves for the 12 day data are shown in Figure 4.6.

12 day chains

The fitted curve of the time course of conversion of the proα1 and proα2 chains closely approximated to the experimental data (Figure 4.6). The fit of the pCα and α curves was less good. The curve for the pNα chains did not fit well to the data. This is not surprising, as very few data points were available and a representative data set could not be obtained.

14 day chains

The fitted curves for the proα1, α1 and all the α2-type chains approximated reasonably the experimental data. The fit for the pCα1 chain was less good and that for the pNα1 chain was not good.

17 day chains

The fit was good for all the chains except for the pNα chains. The fit for the pNα2 chain was reasonable for the early chase time points, but not good after the chase time t=20.

19 day chains

The fitted curves followed the experimental data for the proα1, pCα1 and α1 chains fairly well, but a fit could not be obtained for the pNα1 chain. The observed fit was poor for the proα2 chains, and also for the pNα2 chains at chase times after t=30.

4.6.4.2 Standard Deviation Parameters

The values of the standard deviations of the parameters

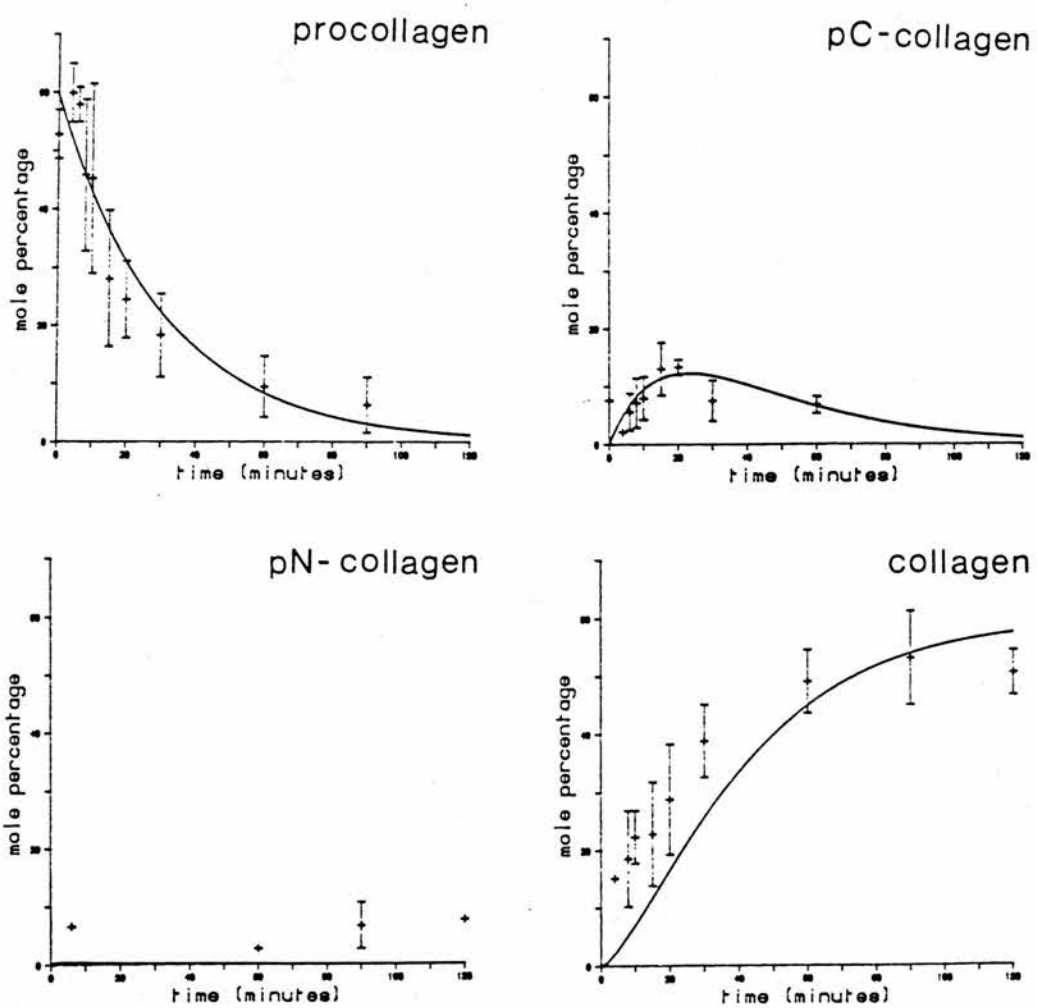

Figure 4.6a Fitted curves for the 12 day α1-type chains. These curves were obtained by using the values of the rate constants in the equations given in Figure 3.4 to generate values of the amount of protein at each chase time point. a). proα1 chain; b). pCα1 chain; c). pNα1 chain; d) α1 chain.

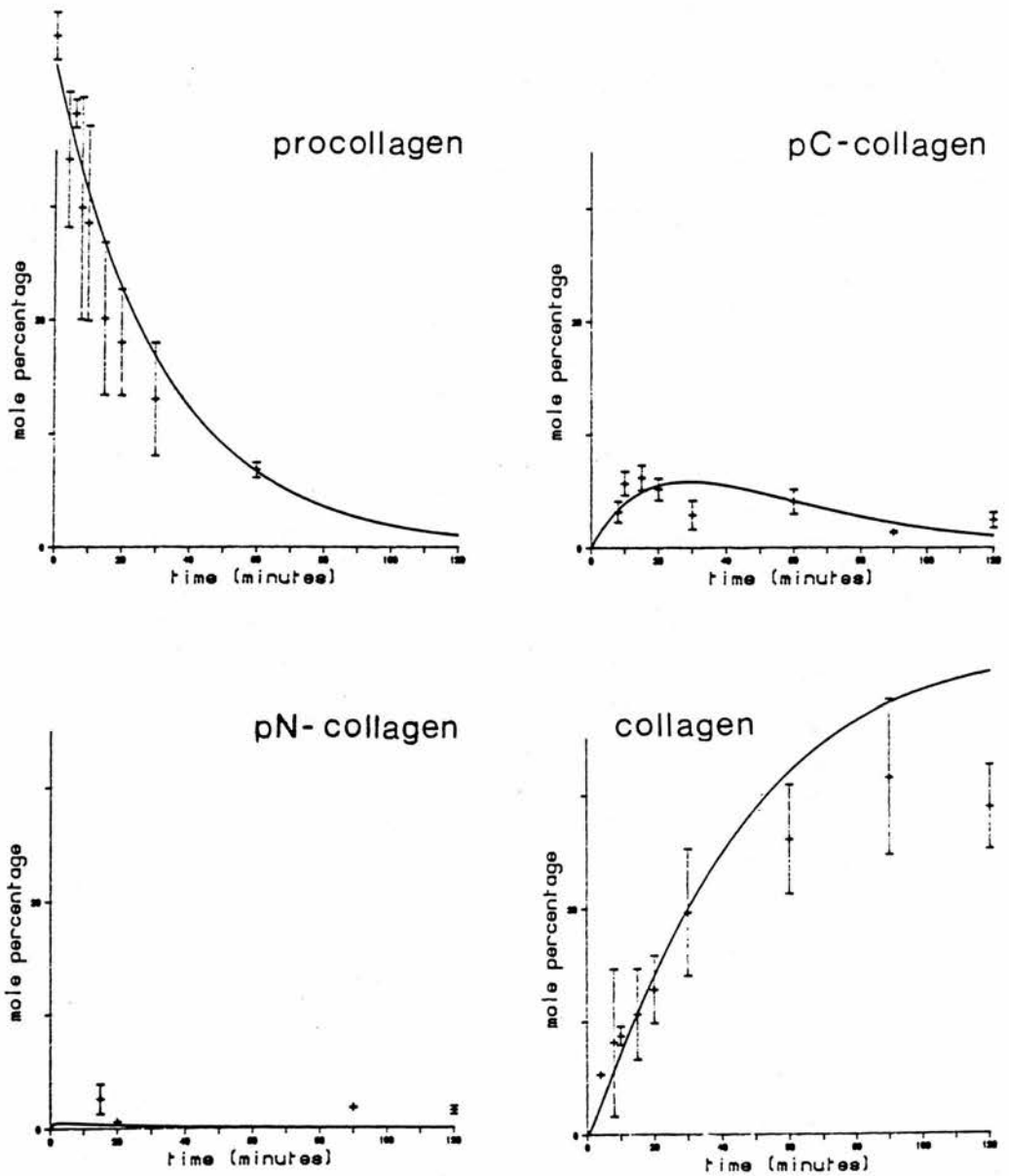

Figure 4.6b Fitted curves for the 12 day α2-type chains. These curves were obtained by using the values of the rate constants in the equations given in Figure 3.4, to generate values of the protein at each chase time point. a), proα2; b) pCα2; c) pNα2; d) α2.

are given in Table 4.1. It was stated in Section 3.6.4 that values of the standard deviations which are greater than 25% are unreliable and an alternative model should be sought. In several cases, the standard deviations of the parameters were greater than 25%. According to the criterion of Section 3.6.4, another model should be sought to explain the data. It is possible that another model is necessary and that the current model is deficient in some respects, but the high standard deviations are probably a consequence of the experimental system. Many of the values of large standard deviations are found for values of k_4. This is obviously a consequence of the small amount of pN-collagen data available for analysis.

4.6.4.3 Residual Plots and Runs Tests

The residual plots of the 12 day α1-type chains and the 17 day α1-type chains are shown in Figures 4.7 and 4.8. These residual plots were selected because they are characteristic of all of the data. If the fit of the curves to the data is good, then approximately equal numbers of positive and negative residuals should be present. Although this situation is obtained in some cases (e.g. 12 day proα1), in many cases there are clusters of positive and negative residuals, which further indicates that the fit may not be good. The clustering was confirmed by carrying out runs tests, in which the number of times that the residuals change their sign was counted. The value of the runs test was always less than 50% (e.g. the 17 day type 2 chains) and in most cases the value was around 30%.

4.6.5 Electron Microscope Data

Samples of tendons at the different developmental stages were fixed in 4% formaldehyde in 0.1 M cacodylate, then processed for electron microscopy (Section 3.9) by Mrs C Cummings and Ms K Greenwood (University of Manchester). The diameters of the collagen fibrils at the three

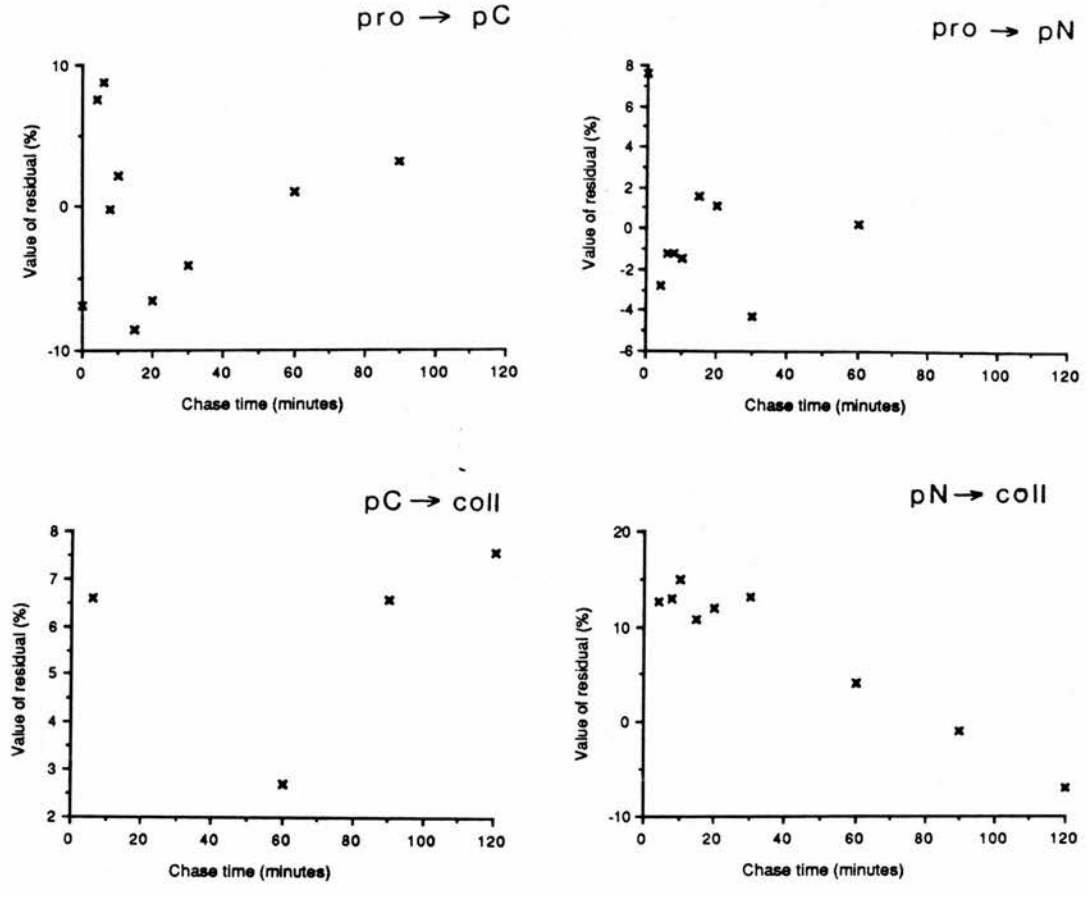

Figure 4.7 Residual plots for the 12 day α1-type chains. The values of the residuals were calculated by the minimization program and then plotted against the chase time to observe the distribution data of the residuals. Ideally, equal numbers of positive and negative residuals should be seen, and these should be evenly distributed throughout the time course of the experiment.

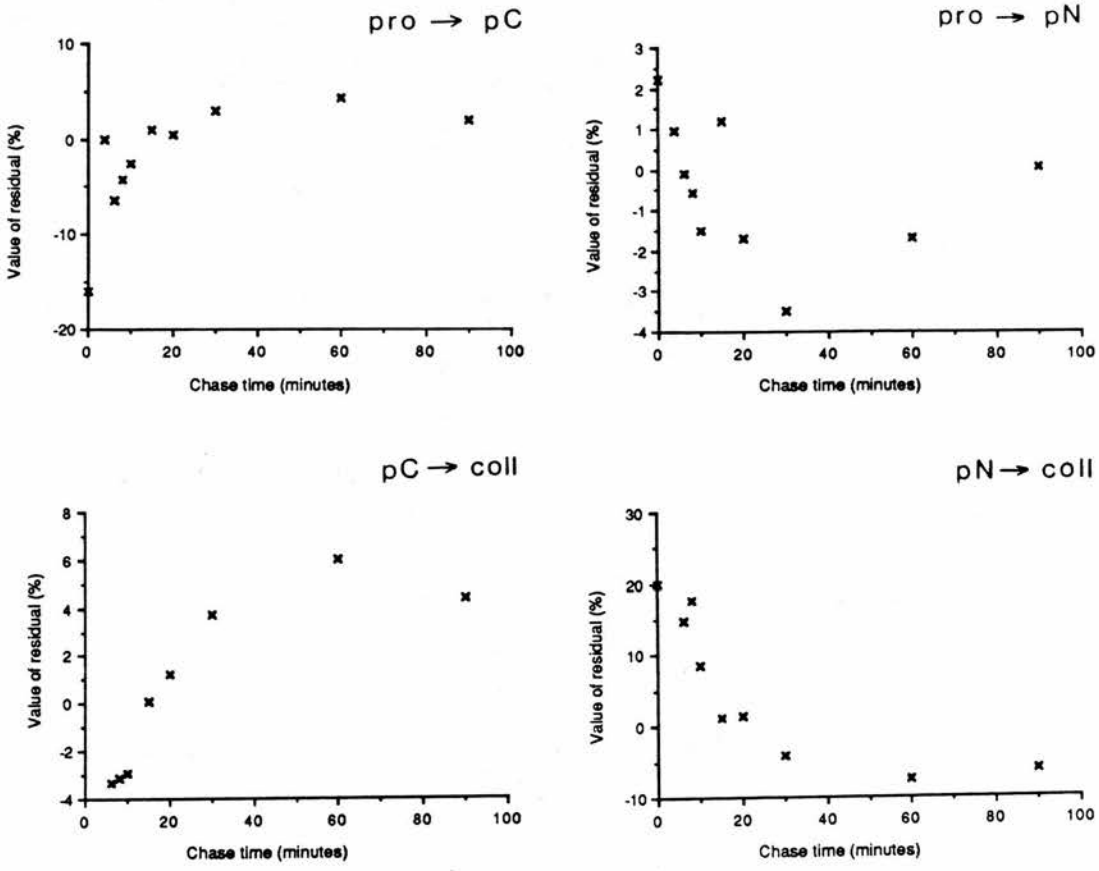

Figure 4.8 Residual plots for the 17 day α1-type chains. The values of the residuals were calculated by the minimization program and then plotted against the chase time to observe the distribution of the positive and negative residuals.

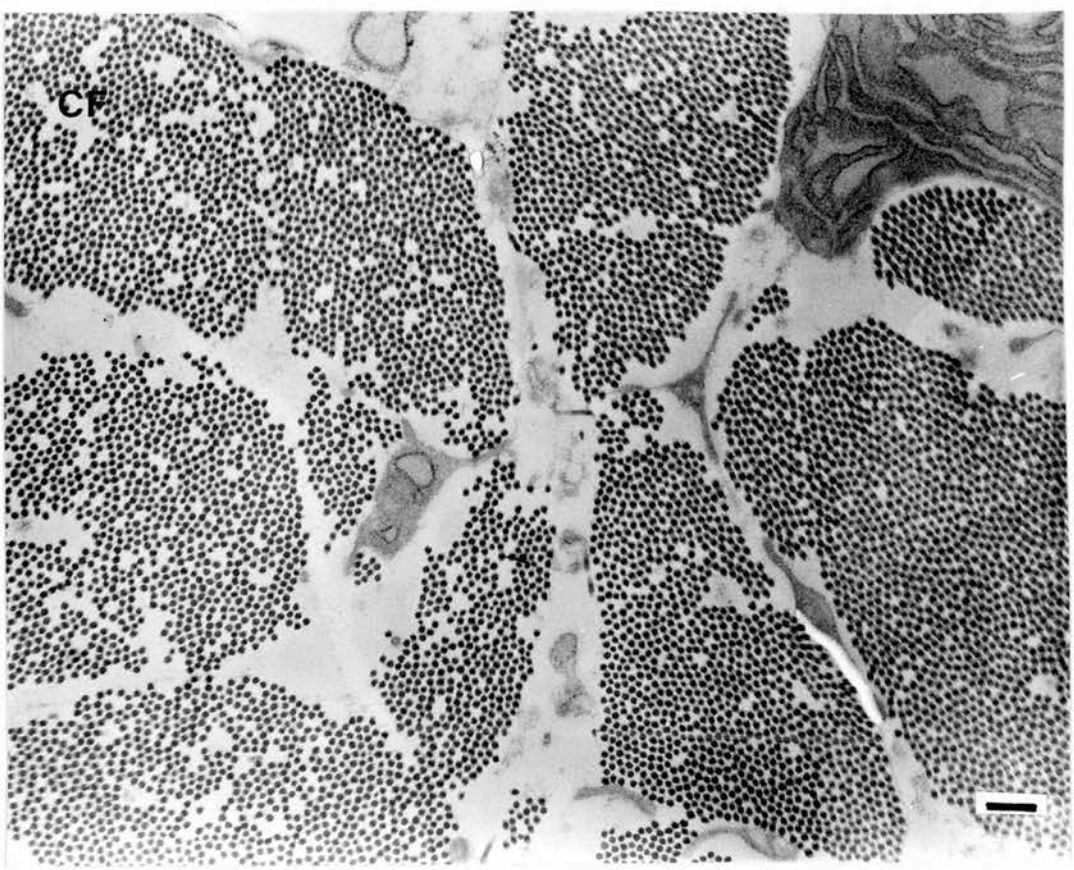

Figure 4.9 A section of a 19 day embryonic chick metatarsal tendon. The tendons were fixed in 4% formaldehyde in 0.1 M cacodylate, stained with 1% PTA + 1% UA, dehydrated in a graded series of ethanol solutions and embedded in Araldite. Courtesy Mrs C Cummings.
Note scarcity of cells and abundance of collagen fibrils (labelled CF). Magnification, x 20,000. Bar, 300 nm. (88E/1183)

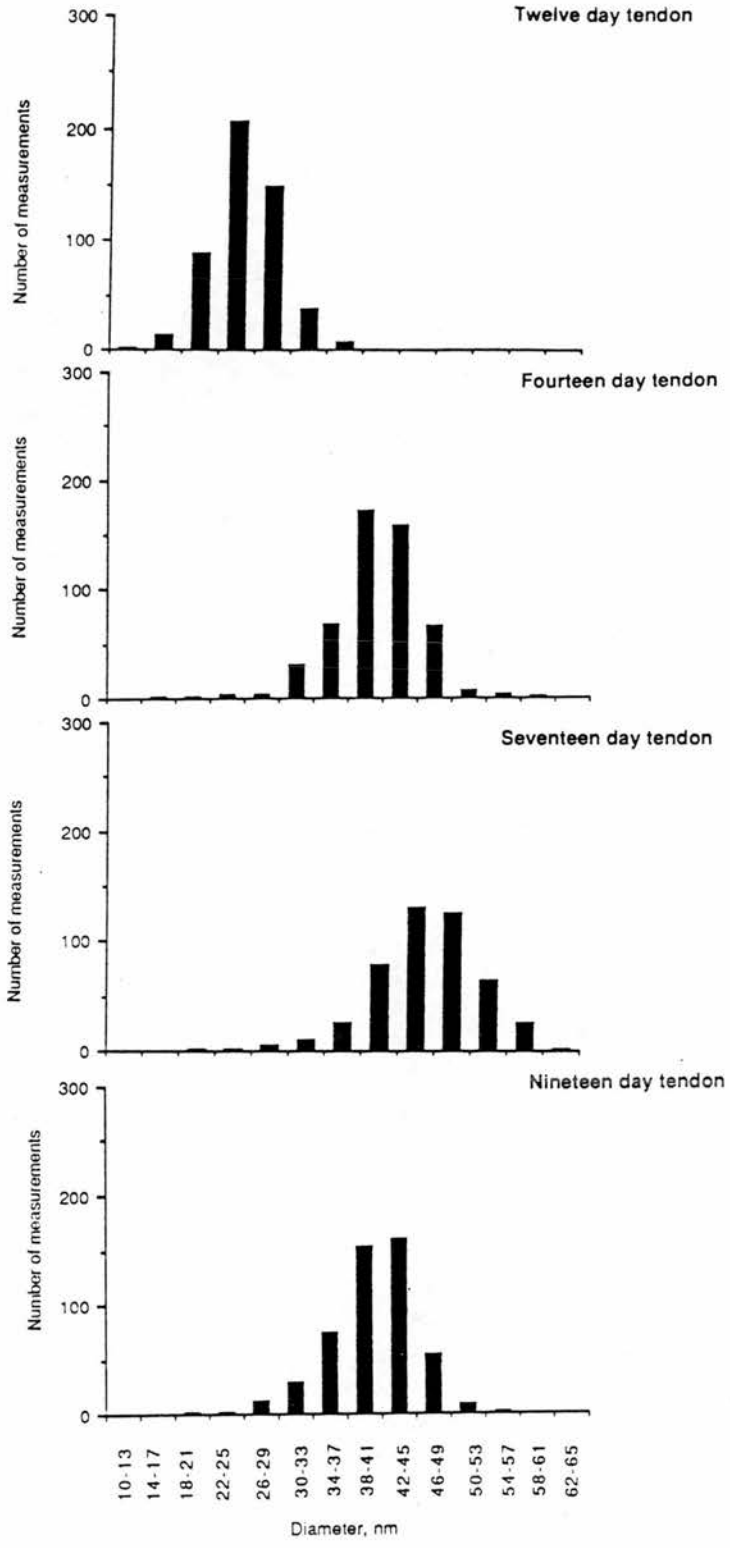

Figure 4.10 Histograms showing the collagen fibril diameter distributions at various stages of tendon development. Note the unimodal distribution and the increasing spread of diameters as development proceeds. Number of measurements: 12 day, 500; 14 day, 498; 17 day, 467; 19 day, 495.

different developmental stages were determined by analysis of the negatives (10,000 x magnification) with a Magiscan 2 (Joyce Loebl). A typical section showing 19-day embryonic chick tendon is shown in Figure 4.9. The data are shown in the form of histograms (Figure 4.10). It can be seen from the histograms that the mean fibril diameter increases from 29 nm to 41 nm between day 12 and day 14, but thereafter there is no significant increase in the diameter of the fibrils.

4.6.6 Efficiency of Extraction

It was important to determine the efficiency of extraction of procollagen, pC-collagen, pN-collagen and collagen by 1 M NaCl, in order to verify that all the collagenous components were extracted with equal efficiency; if one component was selectively extracted over another, this would affect the proportions of the various components determined by densitometry and might affect the parameters obtained by curve fitting. The efficiency of extraction was determined as described in Section 3.7, and the results are shown in Table 4.2.

Figure 4.11 shows typical elution profiles of the separation of the ^{3}H-hypro and ^{3}H-pro in the tissue hydrolyzate and neutral salt extracts for 17 day tendon at chase time t = 180. The efficiency of extraction was determined using the following calculation:

Neutral salt extract:
Total no. hypro counts
= no. counts in hypro peak x a/0.5 x 250/b = A
where a = volume of hydrolyzed sample (ml)
b = volume of original extract taken for hydrolysis (μl)
Thus, a/0.5 is a correction factor for the volume of sample loaded on the column and 250/b is a correction factor accounting for the fact that the collagenous

proteins were extracted in 250 µl of 1 M salt solution, (250-b) µl of which were removed for analysis by SDS-PAGE. For the tissue hydrolyzates,

total hypro = counts in hypro peak x a/0.5 = B

Hence, the efficiency of extraction of collagenous protein = A/(A + B) x 100.

For 17 day tendons at 0 minutes of chase time, 83% of the ^{3}H-hypro-containing molecules were extracted by the 1M salt solution. At 30 minutes of chase time, about 68% of the ^{3}H-hypro-containing molecules were extracted by the 1M salt solution. At 180 minutes of chase time, about 95% of the ^{3}H-hypro containing molecules were recovered in the 1 M salt extract (Table 4.2).

For 12 day tendons at 0 minutes of chase time, the efficiency of extraction (83.8%) could only be determined for one sample. At 30 minutes the efficiency of extraction was about the same (77%), although again only one value could be determined. At t = 180, 82% of the sample was present in the 1 M salt extract.

Although recovery of counts from the Dowex AG50W-X8 column was greater than 90% for the hydroxyproline and proline standards, it was noted that in most cases the recovery of total counts from the column from the samples of the neutral salt extracts and tissue hydrolyzates was much less than this, usually around 60%. There was no correlation with a decrease in recovery of counts with time after preparation of the column. Thus the low recovery of counts was not due to exhaustion of the Dowex column, as replacement of the Dowex AG50W-X8 with fresh resin did not improve the recovery of the column. The low recovery occurred irrespective of whether tissue hydrolyzates or salt extracts were loaded on the column, or whether tendon or corneal samples were used. This low recovery is not seen when ^{14}C-pro and hypro are separated by this technique (McAnulty, personal communication). Thus, the explanation for the low recoveries is not clear. One possibility is that tritium exchange may occur during

	Efficiency of Extraction (%)
12 days	
0	0
	87.3
30	0
	76.6
180	90.8
	74
17 days	
0	78.8
	87.2
30	91.5
	43.8
180	98.2
	91

Table 4.2 Efficiency of extraction of ^{3}H-containing proteins from tendons with 1 M salt at 12 and 17 days of embryonic development. The percentage extraction is shown for two samples at each time point.

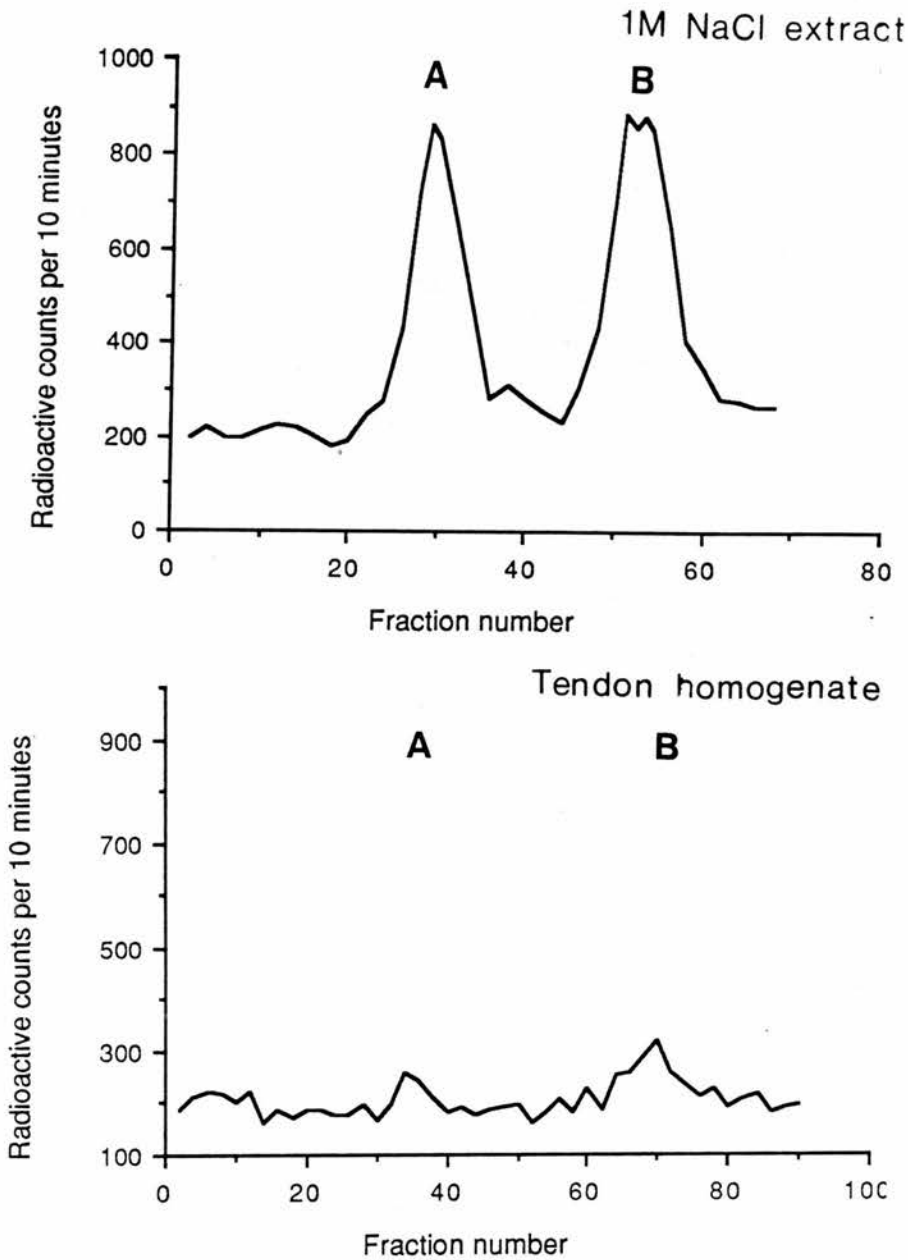

Figure 4.11 Separation of ^{3}H-hypro and ^{3}H-pro in a chase time sample t = 180 for 17 day tendons of the material extracted by 1 M NaCl (Table 3.2) and in the intact tendons after extraction with 1 M NaCl. The extract and the homogenized tissue were hydrolyzed, and the hydrolyzates were applied separately to a Dowex AG50W-X8 column. Fraction volume, 2 ml. Flow rate, 120 ml/hr.

A = Hypro
B = Pro

preparation of the extracts and tissue hydrolyzates- possibly during hydrolysis, as the ^{3}H-hypro and ^{3}H-pro used as standards were not subjected to this step.

4.7. Discussion

4.7.1 Rate constants

It is difficult to draw conclusions about the twelve day data, as the value of k_4 could not be calculated for the α1-type chains. However, the values seem to follow a pattern of random cleavage at either end of the molecule, i.e. k_1 about the same as k_2 and k_4 larger than k_3 (i.e. cleavage of pN-collagen to collagen is rapid). The 14 day data seemed to confirm this pattern of cleavage, as did the 17 day α1-type chains. However, there were two possibilities for the 17 day α2-type chains; either (as above) the first cut at either end of the procollagen molecule was random, or both k_2 and k_4 were less than k_1 and k_3, favouring a model of a processing pathway mainly via a pC-collagen intermediate, in accordance with previous workers (e.g. Morris et al, 1975; Uitto, Lichtenstein and Bauer, 1976; Leung et al, 1979). The values of the min SSQ for the two possible sets of parameters were very similar; hence a distinction between these two possibilities was not possible. It seems unlikely, though, that the pattern of cleavage of the proα2 chain would differ from the proα1 chain.

The 19 day α1-type chain gave similar low k_2-low k_4 values, whereas the α2-type chains showed values all of a similar order of magnitude, with a decrease in k_4 between 17 days and 19 days.

Hence, the majority of the data indicates that the pC-collagen intermediate observed on acrylamide gels in the present study and by previous workers is not due to an exclusive pathway of procollagen processing via a pC-collagen intermediate, but is the result of rapid conversion of the pN-collagen to collagen. Initial removal

of the propeptides is virtually random, i.e. there is no preference for which of the propeptides is removed first. It is clear from this observation, and also from Table 4.1 that the values of k_1 and k_4 are not the same, although these reactions are catalyzed by the same enzyme. Similarly k_2 and k_3 are not the same. C-proteinase catalyzes the conversion of procollagen to pN-collagen, and pC-collagen to collagen (k_2 and k_3 respectively) while N-proteinase catalyzes the reactions pro to pC and pN to collagen (k_1 and k_4 respectively). Thus, it seems surprising that the values of the rate constants of the reactions which result in removal of the same propeptide are not the same. One hypothesis is that the substrate cleavage site is being presented to the cleaving enzymes in a different way. It could be suggested that the pN-collagen molecules are aggregating, and that the N-proteinase prefers to act on an aggregated substrate and thus the rate constant for N-proteinase increases.

In a minority of cases a (low k_2, low k_4) situation appeared to exist, or could not be distinguished from the (random cleavage, high k_4) model. The standard deviation parameters were unfavourable in the low k_2, low k_4 model. Thus it is postulated that the program could not distinguish between the two models because of poor data with large scatter. Better data would clarify the situation. However, the data obtained are the best possible considering the experimental design and collection of data. The wide scatter is a consequence of the way the data are obtained. Thus, in such cases where regression analysis is used to fit curves to data with a wide degree of scatter, it is recommended that a global search is carried out to determine all possible values of the rate constants.

4.7.2 Variation During Embryonic Development

It must be borne in mind that the parameters obtained in

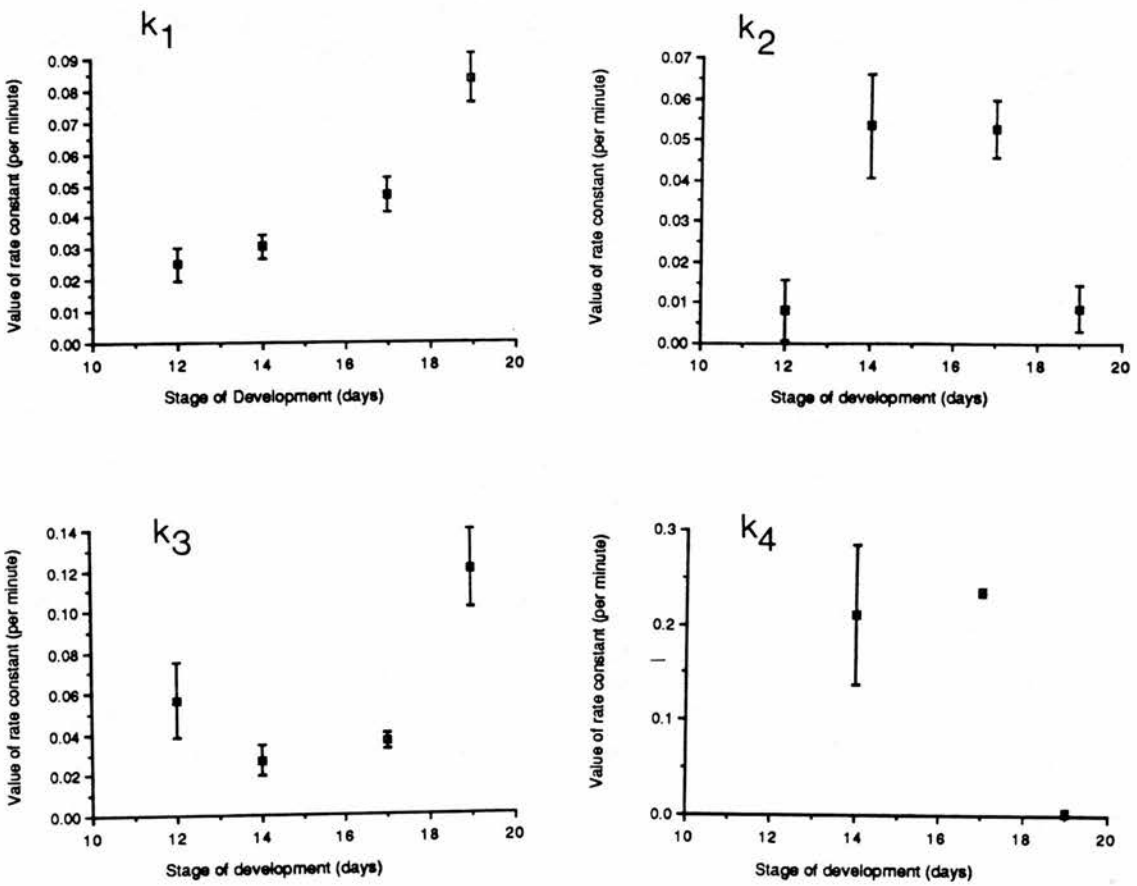

Figure 4.12 Variation of the rate constants with development (α1-type chains).

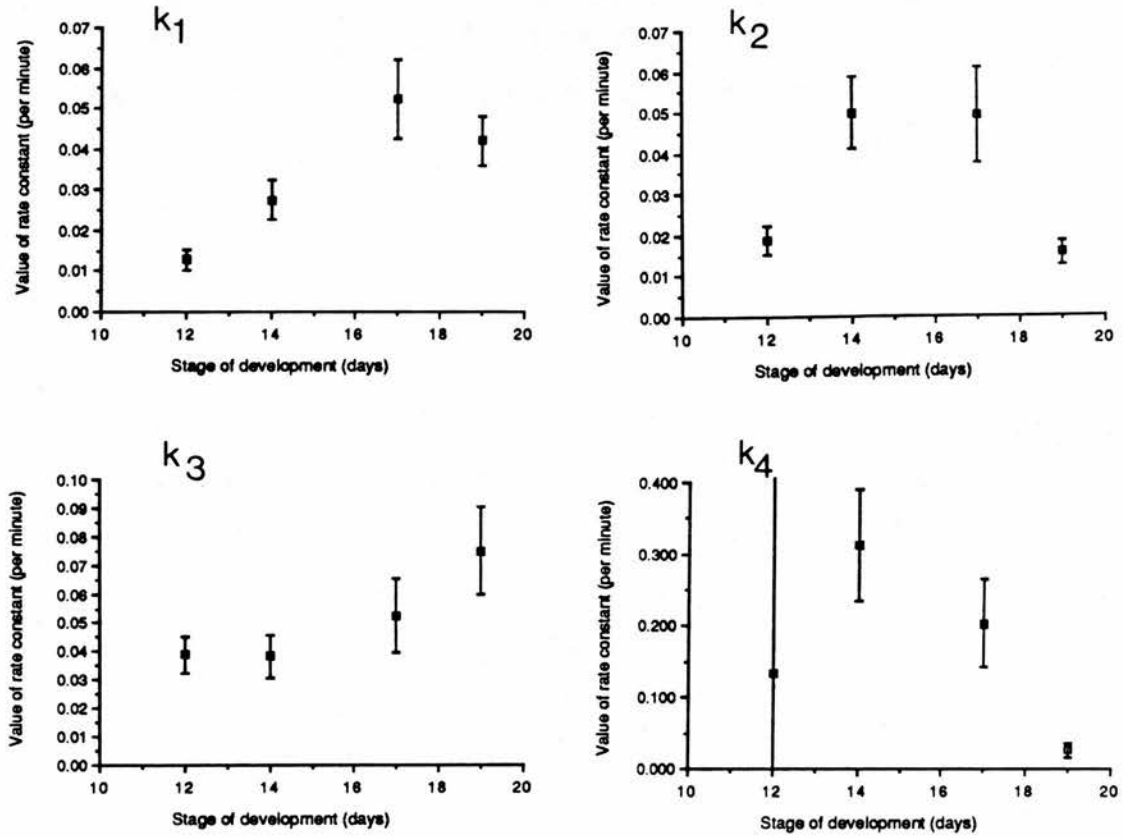

Figure 4.13 Variation of the rate constants with development (α2-type chains).

this study are not rates, but rate constants. These rate constants are related to the rate by the expression

rate = concentration of a component x rate constant

As the concentrations of procollagen, processing intermediates and collagen were not calculated in this study, it is not possible to give values for the actual rates of the reactions.

The variation of the rate constants during the developmental stages studied is shown in Figure 4.12 for the α1-type chains and Figure 4.13 for the α2-type chains. For the α1-type chains, k_1 increases between 12 and 19 days of development. The value of k_2 increases significantly between 12 and 14 days of development. The value of k_3 increases between 17 and 19 days. The data for the α1-type chains at 19 days must be treated with some caution, as the data follow a model of (low k_2, low k_4), and thus the values of the rate constants do not relate to the 12, 14, and 17 day data which follow a (random first cleavage, large k_4) model. A value of k_4 could not be obtained for the 12 day α1-type chains. The value of k_4 does not change between 14 and 17 days of development.

For the α2-type chains, k_1 increases between 12 and 17 days, and k_2 increases between 12 and 14 days. The values of the min SSQ for the 17 day α2-type chain are consistent with either initial random cleavage or a (low k_2, low k_4) model. If the random cleavage data are compared with the rate constants for 12 and 14 days, it can be seen that k_3 for the α2-type chain does not increase significantly at the stages of development studied (Figure 4.13). If the data for the 17 day α2-type chain which are consistent with a random cleavage model are compared with the 12 day and 14 day data, it can be seen that the value of k_4 is about the same as at 12 and 14 days of development, but this decreases to a very low value at 19 days. It is not clear why this should be the case. As observed earlier, the 19 day data do not fit the pattern of the data obtained at earlier stages and thus a direct comparison of

the change of the rate constants between the earlier developmental stages and 19 days is not possible.

It seems from the data that the rate of processing could be increasing or remaining constant during embryonic development, and it is interesting to compare the current data with results obtained by previous workers. Data were obtained for the amounts of hydroxyproline in chick tendons by Scott and Hughes (1986). The amount of hydroxyproline (mg/g dry weight tendon) increased from 4.9 at 11 days to 16 at 14 days; 43.5 at 17 days and 55.4 at 19 days. As development proceeds, the fibril packing fraction (i.e. the volume occupied by the collagen, not the cells) increased (Fitton Jackson, 1956; McBride, Hahn and Silver, 1985). Thus it seems clear that with development the overall amounts of collagen increase. This could be explained either by a constant rate of processing, or an increased rate of processing.

It is interesting to note that prolyl hydroxylase activity is at a maximum in late log phase in cell cultures (for references, see Cardinale and Udenfriend, 1974). Collagen and prolyl hydroxylase were synthesized at a time when cellular protein synthesis was at a maximal rate. Activity of lysyl oxidase was also found to be at a maximum at the end of the log phase (Layman, Narayanan and Martin, 1972). A crude preparation of "procollagen peptidase" (presumed to be procollagen N-proteinase) was also maximally active at this time (Layman and Ross, 1973).

In vivo or in vitro administration of dexamethasone to embryonic chicks or embryonic chick sterna had no effect on the conversion of type II procollagen to collagen, despite a decrease in the rate of collagen synthesis and a slight decrease in the amount of prolyl hydroxylation (Oikarinen et al, 1988). Also, in chick osteoblast culture, although collagen was accumulating, the synthesis rate was decreasing (Gerstenfeld et al, 1988) and the extent of procollagen processing increased with days in

culture. However, it is not clear how the data obtained in cell culture systems (Layman, Narayanan and Martin, 1972; Layman and Ross, 1973; Gerstenfeld et al, 1988) could relate to the tissue situation.

4.7.3 Discussion of Electron Microscope Data

The EM data contrast with those of Fitton Jackson (1956), who obtained values for the fibril diameters of 17 nm at 12 days, 25 nm at 14 days, and between 31-40 nm at 17 days. EM data of Eikenberry et al (1982) show diameters of 39 nm (12 days), 40 nm (14 days) and 41 nm (17 days). The mean diameters at 14 days and 17 days are very similar to those obtained in the present study (41 nm at 14 days, 45 nm at 17 days). In this study, the diameter at 19 days was 41 nm; diameters at this stage were not measured by Eikenberry et al (1982) in the EM, but their value for 18 day tendon was 52 nm, indicating that a further increase occurred between 17 and 18 days. At stages later than 17 days in the study of Eikenberry et al, a multimodal distribution was obtained, whereas in the current study only a unimodal distribution was seen. Scott and Hughes (1986) obtained values for the fibril diameters of 25 nm (11 days); 35 nm (14 days); 45 nm (17 and 19 days). The spread of the diameters from this study is not known; hence it is not clear if the increase in fibril diameter between these stages is significant.

The range of diameters observed here was 12-40 nm at 12 days, 14-58 nm at 14 days; 20-62 nm at 17 days; and 18-58 nm at 19 days. Hence, the range of diameters does not appear to change between 14 and 19 days of development. This is in contrast to Eikenberry et al (1982), where the range of fibril diameters was 24-52 nm at 14 days and 16-74 nm at 18 days of embryonic development. Eikenberry et al (1982) suggested that as growth proceeds the broader distribution of sizes could result from using the existing fibrils as a scaffold.

An X-ray study (Eikenberry, Brodsky and Parry, 1982) gave values for the fibril diameters of 48 nm at 14 days, 57 and 58 nm at 17 days and 18 days respectively. These values would be for fully hydrated samples, whereas the EM values were obtained from samples dehydrated during preparation for EM. Their X-ray data showed an increase between 14 and 17 days of development, but were constant after that. The present data showed no change between 14 and 19 days.

Thus, the mean values of the fibril diameters obtained by EM in the present study are similar to those obtained by Eikenberry et al (1982), but differ from those of Fitton Jackson (1956).

4.7.4 Correlation with fibril diameters

For all cases except 17 day α2-type chains and 19 day α1-type chains, the fitted curves and values of the parameters show conclusively that the initial cleavage at either end of the procollagen molecule is random, i.e. there is no preferred order of processing. Determination of the fibril diameters showed an increase in the mean diameter between 12 and 14 days of embryonic development, but no increase in the diameters after this stage. It might have been expected that if procollagen processing played a role in the regulation of fibril diameter that the rate of the reaction pro to pC might have increased relative to pro to pN if pC was involved in the regulation of wide diameter fibrils. No such change was apparent. Also, the fact that the pathway of processing involves random cleavage precludes a role of the intermediates in regulation of diameter.

4.7.3 Efficiency of extraction

It was shown that at chase times 0, 30, 180 at 12 and 17 days of embryonic development, most of the ^{3}H-labelled

hydroxyproline-containing protein was extracted by the 1 M salt solution. The fact that most of the hypro was extracted indicates that a) there was no preferential extraction of one collagenous species over another b) secretion was occurring at the chase time t = 0, that is about 20 minutes after the addition of the radioactive label (Harwood, Grant and Jackson, 1976; Kao, Berg and Prockop, 1977; Kao, Prockop and Berg, 1979; Kao, Mai and Chou, 1982; Kao et al, 1983).

CHAPTER 5

PROCOLLAGEN PROCESSING IN THE CORNEA

5.1 The Cornea

Apart from being the most important refractive medium in the eye because of the difference between the refractive index of cornea and air (Maurice, 1969), the cornea must also be tough to withstand intra-ocular pressures and to protect the eye from mechanical injury (Maurice, 1957). The arrangement of the collagen fibrils in the cornea is thought to account for its transparency (Maurice, 1957; 1969). The fibrous arrangement also accounts for its mechanical properties. Collagen accounts for 70% of the dry weight of the cornea (Maurice, 1969).

In this section, reference will be made mainly to the avian cornea. There are close functional and structural similarities between the corneas of birds and primates (Renard et al, 1978). Reviews of the fine structure of the cornea have been written by Tripathi (1974), Marshall and Grindle (1978) and Freeman (1982a).

The avian cornea consists of five layers (Figure 5.1). The outer surface is a multilayered stratified non-keratinized squamous epithelium derived from the ectoderm. The second layer is Bowman's membrane, or Bowman's layer. It consists of a network of types I and II collagen (von der Mark et al, 1977; Hendrix et al, 1979). The thickest layer of the cornea is the stroma, which is largely acellular and accounts for about 90% of the total thickness of the cornea at hatching. The stroma consists of a highly organized network of collagen fibrils arranged in layers orthogonal to one another, proteoglycans, and fibroblastic cells known as keratocytes. In human cornea the keratocytes constitute only 2-3% of the volume of the stroma (Tripathi, 1974; Marshall and Grindle, 1978). The lamellae of collagen fibrils in the adult chicken are not strictly orthogonal to each other, but are displaced by a few degrees (Hay, 1980). The fourth layer of the cornea is Descemet's membrane, the basement membrane of the corneal endothelial cells (Freeman, 1982a). Descemet's membrane

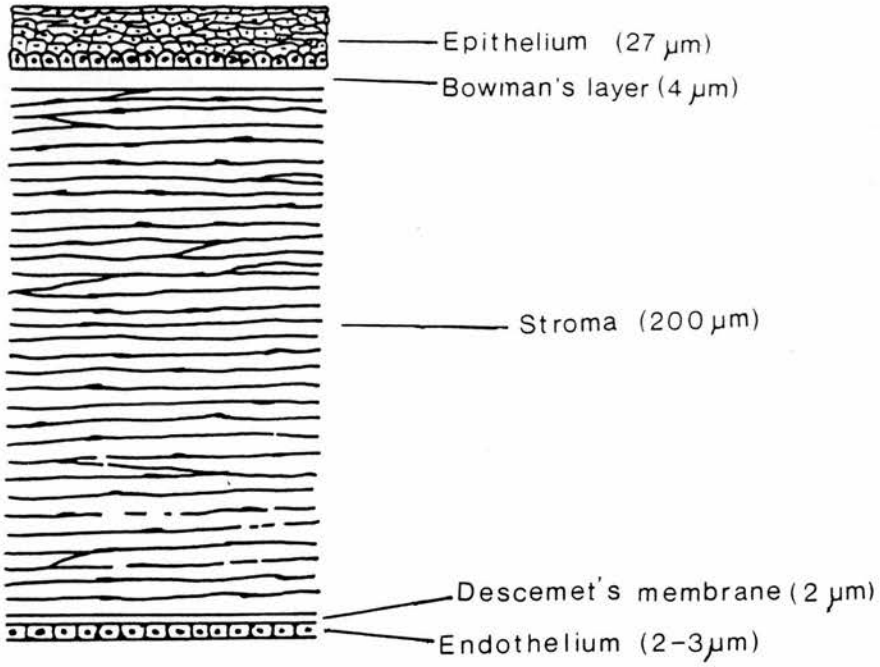

Figure 5.1 Schematic representation of a section through the chick cornea (adapted from Harding, Crabbe and Panjwani, 1980). The figures in brackets refer to the dimensions of the various layers after hatching (taken from Hay and Revel, 1969). The thickness of Descemet's membrane increases with age (Freeman, 1982a).

contains type VIII and IV collagen. The structure of type VIII collagen is thought to explain the characteristic nodular arrangement of collagen seen in Descemet's membrane (Labermeier and Kenney, 1983; Bornstein and Sage, 1987). The innermost layer of the cornea is the endothelium which consists of a single layer of cells (Freeman, 1982a). The dimensions of the various layers after hatching are shown in Figure 5.1.

The collagen fibrils of the cornea in the adult chicken are narrow and of a uniform diameter (24 nm) (Craig and Parry, 1981). They are spaced regularly in the stroma equidistant from each other in a GAG-rich matrix. The properties of the highly charged GAG chains have been proposed to account for the precise spacing (see references in Scott and Haigh, 1988). Such a role may be more likely for CS-DS-PG than KS-PG, as there is virtually no KSPG in the stroma of the mouse (Scott and Haigh, 1988). It is possible that the fibrils are coated in the GAG-PG such that the negative charges of the GAG chains mutually repel one another, resulting in the equidistant spacing of the fibrils. However, removal of the extractable GAG had no effect on the stromal organisation or spacing of the fibrils (Dische, Cremer-Bartels and Kaye, 1985; Bard, Bansal and Ross, 1988; Bansal, Ross and Bard, in press). Maurice (1957, 1969) proposed that such an arrangement of fibrils would act as a diffraction grating, so that all of the scattered light is suppressed by destructive interference, leaving only the recombined rays in the direction of the original pathway of the beam, if the distance between the fibrils is smaller than the wavelength of light. The beam of light will be transmitted unchanged by the "grating", so the cornea will appear transparent.

In the chick, adult levels of tranparency and light transmission develop by 19 days <u>in ovo</u>. The amount of light transmitted increases from 14-19 days of development, correlating with a gradual loss of water from

the cornea (Coulombre and Coulombre, 1958). The loss of water might result in greater ordering of the fibrils in a lattice array. For the array to be maintained, the cornea must be able to resist swelling pressure, possibly by constant pumping out of water (Masterson, Edelhauser and Van Horn, 1977).

An interesting question in collagen fibrillogenesis is how the orthogonal arrangement of fibrils is laid down in the cornea. Orthogonal fibrillar arrangements have occasionally been observed when fibrils are reconstituted by neutralizing and warming collagen solutions to 37°C (Trelstad, Hayashi and Gross, 1976; Bouligand et al, 1985). This indicates that collagen can self-assemble into orthogonal arrays without the influence of cells (Bouligand et al, 1985). In contrast, Birk and Trelstad (1984) claimed that orthogonality is directed by the corneal fibroblast. In chick corneal fibroblasts, these authors observed small recesses containing 5-12 fibrils and fusion of two or three recesses with coalescing of the fibrils to form small bundles which contained 50-100 fibrils. The surface foldings fused, then receded and the surface retracted to form lamellae. Birk and Trelstad (1984) claimed that the corneal fibroblast had an orthogonal configuration when studied in thick sections by high voltage electron microscopy.

5.2 Development of the Avian Cornea

A brief outline of the development of the avian cornea will be given here. For greater detail and further references, the reader is referred to the excellent monograph by Hay and Revel (1969) and the review by Hay (1980).

It should be borne in mind that the development of the avian cornea differs from the development of primate and rodent cornea in several aspects (Hay, 1980) and these will not be further discussed.

In the chick embryo, optic vesicles appear as lateral expansions of the forebrain at 30 hrs post-fertilization (stage 9; Table 5.1) and become constricted at their bases by 40 hrs. By 48-52 hrs (stage 13; Hamilton, 1952) the ectoderm overlying the optic vesicle has thickened to become the lens placode. The optic vesicle then starts to invaginate and the lens placode follows its contour, and becomes fully invaginated to form the lens by 3 days of development. The lens vesicle then detaches from the overlying ectoderm, which is now the corneal epithelium. Between stages 18-28, the cells of the epithelium (2 cells thick) hypertrophy, and acquire secretory apparatus. By the end of stage 18 the first fibrils of the primary stroma have appeared between the epithelium and the lens, produced by the epithelium (Hay, 1980; Hayashi et al, 1988). The epithelium produces both types I and II collagen and the endothelium can also synthesize small amounts (Hayashi et al, 1988). At stage 22 (3.5 days) endothelial cells probably derived from the neural crest (secondary mesenchyme; see Hay, 1980, for references) migrate between the stroma and lens. Light and electron microscopy indicates that both the primary stroma and lens can act as substrata for the migrating endothelial cells. As the cells migrate, they flatten out and move along the inner surface of the primary stroma. The endothelial cells have formed a simple cuboidal confluent layer by stage 27-28 (5.5-6 days), and this is accompanied by a swelling of the stroma. This swelling occurs just prior to the invasion of fibroblasts from the secondary mesenchyme of the neural crest. The stroma swells from 12-30 μm in the latter half of stage 28, and swells to 60 μm by the end of stage 28 (Trelstad and Coulombre, 1971). This sudden swelling is also accompanied by the formation of tight junctions between the endothelial cells. Invading fibroblasts are fully differentiated to form fibroblasts by stage 28, and first move into the area of the stroma near the endothelium. The posterior region of the stroma

Stage	Days	Stage	Days
1-5	to 22 hours	26	4.5-5
6	23-25 hours	27	5
7	23-26 hours	28	5.5
8	26-29 hours	29	6
9	29-33 hours	30	6.5
10	33-38 hrs	31	7
11	40-45 hrs	32	7.5
12	45-49 hrs	33	7.5-8
13	48-52 hrs	34	8
14	50-53 hrs	35	8-9
15	50-55 hrs	36	10
16	51-56 hrs	37	11
17	52-64 hrs	38	12
18	65-69 hrs	39	13
19	68-72 hrs	40	14
20	70-72 hrs	41	15
21	3.5 days	42	16
22	3.5 days	43	17
23	3.5-4 days	44	18
24	4 days	45	19-20
25	4.5	46	20-21

Table 5.1 Stages in the development of the chick embryo (after Hamburger and Hamilton, 1951; Hamilton, 1952)

is transformed by the keratocytes, which lay down more collagen, using the existing lamellae of the primary stroma as a framework (Hay and Revel, 1969). Primary stroma continues to be laid down until stage 36 (10 days), with the newest layers being deposited nearest the epithelium, so that the deepest layers are the oldest (Trelstad and Coulombre, 1971; Hay, 1980). By stage 36 the uninvaded primary stroma is 1 μm thick. The primary stroma contains 30 collagenous lamellae orthogonally arranged, each 1 fibril thick (Hay and Revel, 1969). At the end of stage 27 (when the first fibroblasts invade) there are about 20 lamellae of collagen fibrils in the anterior stroma and 10 in the posterior stroma. By stage 30 there are 15 layers of uninvaded fibrils and 12-15 layers of fibroblasts-this is approximately the number of lamellae now invisible, which suggests that these layers have acted as a scaffold. Micrographs have been obtained showing fibroblasts between the lamellae (Hay and Revel, 1969).

The first layers of the primary corneal stroma formed during days 3-5 of development are deposited in a strict orthogonal pattern. By stage 29 the stroma has swollen to 110 μm thick, with only the anterior 10 μm uninvaded by mesenchyme. The orthogonal layers of collagen are not displaced. However, in the anterior uninvaded portion, the near layers are angularly displaced by 2°. At day 9 the posterior 140 μm of stroma show no displacement, but at day 14 only the posterior 50 μm show no displacement, the difference resulting from compaction (Trelstad and Coulombre, 1971). The fibrils appear to form bundles which are 1-3 fibrils thick and 5-25 fibrils wide (Trelstad and Coulombre, 1971). The distance between fibrils in a bundle is 60 nm, but between adjacent fibrils the spacing is not uniform and ranges from 30 nm to 100 nm.

A recent study has shown that the chick primary corneal stroma may not be as organized as previously thought (Bard and Bansal, 1987). At stage 24 the fibril diameters are uniform (20.5 nm). Foetal corneas are thought to have

diameters of 17 nm (Craig and Parry, 1981) but this is using dehydrated samples. Although by stage 26 most of the newly laid down fibrils have uniform diameters, the collagen near the endothelium has a mean diameter of 22.8 nm, some of the fibrils have much wider diameters (40 nm) (Bard and Bansal, 1987). Thus while most collagen forms fibrils of 20 nm, some of the collagen molecules enlarge existing fibrils by diffusion at a rate of 0.1 nm/hr. Scanning electron microscopy showed that orthogonality exists only in short range order-only a few fibrils are parallel to one another in any one plane before the array is broken by orthogonal fibrils (Bard and Bansal, 1987). Thus the primary stroma may not be dictating the structure of the secondary stroma, and this is supported by the fact that there is very little organized primary stroma in humans, and none in rodents (Hay, 1980).

An inhibitor of GAG synthesis, 6-diazo-5-oxo-L-norleucine (DON) was injected into the chorioallantoic circulation of 5 day old embryos, just before the layers of the primary stroma became rotated (Coulombre and Coulombre, 1975). The axes of the fibrils deposited immediately following the application of DON were rotated in the opposite direction, and usually rotated normally after DON had become inactive. The invading fibroblasts copied the abnormal rotation of the affected fibrils of the primary stroma (Coulombre and Coulombre, 1975).

At 9-10 days (stage 35-36) stratification of the epithelium begins. At stage 36 there are about 20 layers of cells in the stroma and 25 by day 14, and this is similar to the number in the hatched chick. Descemet's membrane first appears by stage 35 and is produced by the cells of the endothelium.

Condensation of the stroma begins at stage 40 (day 14) and the posterior stroma condenses first (Hay and Revel, 1969). The cornea loses water rapidly, resulting in closer packing and a more orderly arrangement of the fibrils, acquiring adult levels of transparency by day 19

(Coulombre and Coulombre, 1958). The water content decreases from 90% at day 9 to the adult level of 76% at 19 days.

Thyroxine has been found to accelerate the development of the endothelium if applied at stage 36 or 38 by accelerating the development of endothelial interdigitations (Masterson, Edelhauser and Van Horn, 1977). These normally develop between stages 38-45. Thiouracil delayed the development of the interdigitations, and the thiouracil-treated cornea remained swollen, whereas the thyroxine treated cornea condensed sooner. The junctions which make up the epithelium are tight junctions, and thiouracil delayed the development of the epithelium. Thyroid hormone could be enhancing the ability of the endothelium to pump water out of the cornea (Masterson, Edelhauser and Van Horn, 1977).

5.3 Corneal collagen

The collagen fibrils in the stromas of all vertebrate corneas have been shown to have a narrow range of diameters with a mean diameter of about 25 nm in most cases (Craig and Parry, 1981). The cornea also has a structural and protective role and the collagen of the cornea might be important in this respect. Linsenmayer, Gibney and Fitch (1986) used immunofluorescence to show that there were bands of corneal collagen extending across the whole width of the cornea which apparently had greater thermal stability than most of the corneal collagen. These bands might be important in stabilizing the shape of the cornea (Linsenmayer, Gibney and Fitch, 1986).

Corneal and scleral type I collagen molecules had the same translational diffusion coefficient, particle scattering factor and molecular length (Birk and Silver, 1983). SLS crystallites prepared from the two collagens were similar, suggesting that the charge distributions on the two molecules were similar. The data of Birk and

Silver did not show significantly different physical properties. These studies were carried out at pH 2, so interactions would possibly be different in a physiological solution. The type I collagen of the cornea is more glycosylated than other type I collagens (Grant et al, 1969; Schofield, Freeman and Jackson, 1971; Harding, Crabbe and Panjwani, 1980; Kao et al, 1983). Corneal type I procollagen is secreted about 25 minutes after synthesis begins, similar to tendon type I procollagen which is secreted after 20 minutes (Kao, Mai and Chou, 1982). The amounts of proline and lysine hydroxylation in corneal and tendon procollagen are the same, and the rates of chain association and secretion in the two tissues are not significantly different (Kao et al, 1983).

Corneal epithelia synthesized type I and a trimer of an α chain, which could possibly be type IV (Trelstad, Hayashi and Toole, 1974). However, the collagen showed all the characteristics of type II based on elution from CM-cellulose (Linsenmayer, Smith and Hay, 1977). Isolated stroma synthesized only type I collagen (Trelstad, Hayashi and Toole, 1974). Kao, Mai and Chou (1982) found that 5% of the corneal collagen synthesized could be attributed to type V; less than 1% of the tendon collagen was type V. Pöschl and von der Mark (1980) found that 20% of the 14 day collagen could be attributed to type V. Results from cultured human stromal fibroblasts indicated that essentially all of the collagen synthesized is type I (Stoesser, Church and Brown, 1978).

In the Mov13 mouse, no type I collagen is synthesized. Fibroblast invasion occurs normally in the mouse embryo, at 12 days of development, but very little collagen is present in the stroma, and that which is present is not orthogonally organized (Bard and Kratochwil, 1987). This suggests that type I collagen is necessary for the orthogonal organization, and that other collagen types cannot compensate for the deficiency in type I collagen, or increase their synthesis. This is surprising in view of

the fact that other fibrillar collagens are present in the cornea of the mouse (Harnisch et al, 1978; Bard and Kratochwil, 1987) and that in the chick types I and V form copolymeric fibrils (Birk et al, 1988) and that type V collagen alone can form thin fibrils in vitro (Broek et al, 1985; Adachi and Hayashi, 1986).

The amino acid compositions of avian corneal and tendon type I collagen were found to be very similar (Trelstad and Kang, 1974). Amino acid compositions of human corneal, scleral and skin type I collagens also showed a very high level of homology, with differences in the hylys contents (Freeman, 1982b). The corneal collagen migrated more slowly on gels, which could be attributed to differences in the level of post-translational modifications. Protease cleavage patterns were similar for the three type I collagens.

In contrast, differences in the avian and tendon corneal type I collagen peptide maps have been reported (Kao and Foreman, 1980). Differences were greater for the $\alpha 2$ than the $\alpha 1$ chain. Further investigations using peptide maps of tendon and corneal unhydroxylated procollagens showed that there were no differences between the pro$\alpha 1$(I) chains of the two tissues, but the pro$\alpha 2$(I) maps differed. The unhydroxylated $\alpha 1$(I) and $\alpha 2$(I) maps were identical in this system, so the differences could be due to differences in the propeptides (Kao, 1985).

5.3.1 Collagen Synthesis and Collagen Types During the Development of the Cornea

Collagen synthesis was found to increase between days 9-20 of development, with most rapid increases between 15-20 days (Figure 5.2). Collagen increased from one-third of the non-collagen protein level at 9 days, to three times the level at 20 days. The rate of synthesis decreased slightly at 1 day post-hatching and then recovered and continued to increase (Coleman, Herrmann and Bess, 1965).

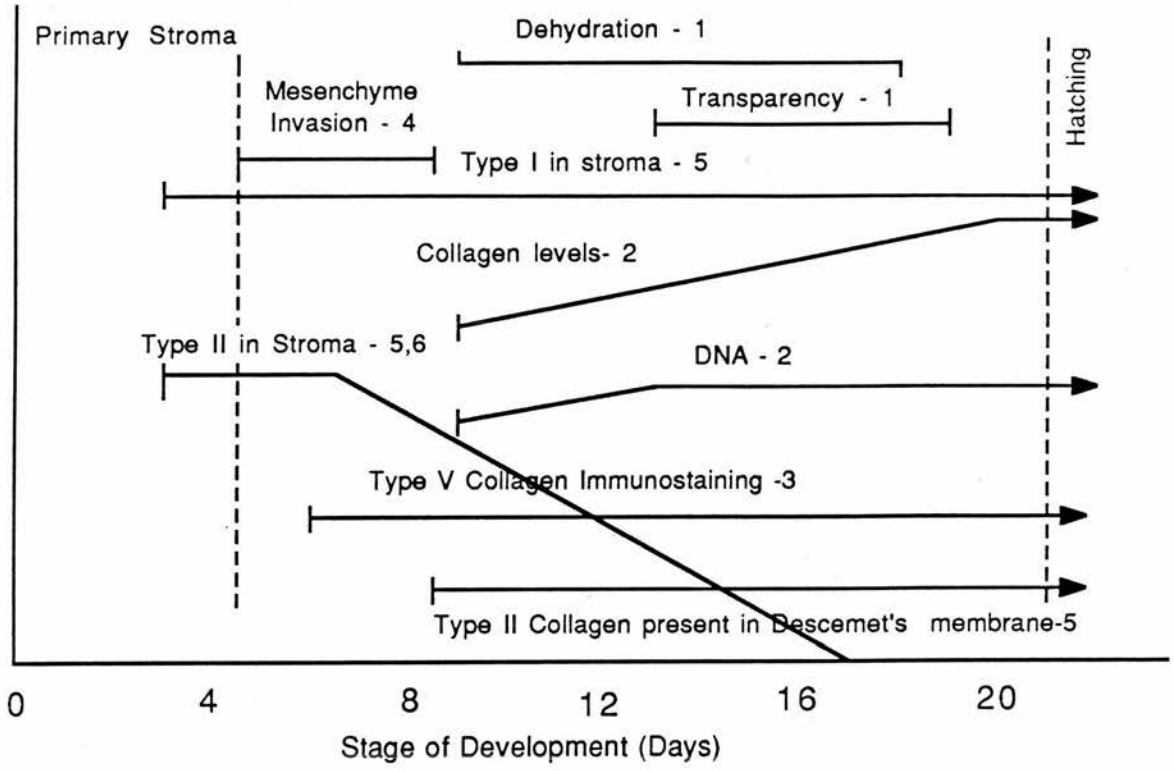

Figure 5.2 Diagram of the changes that occur in the synthesis of collagen types during the development of the chick embryo. The x axis shows the days of development. The changes in synthesis of the collagen types are shown in relation to the major events in corneal morphogenesis: the formation of the primary stroma, mesenchyme invasion and the development of dehydration and transparency.

The numbers refer to the following papers: 1. Coulombre and Coulombre, 1958; 2. Coleman, Herrmannn and Bess, 1965; 3. Linsenmayer, Fitch and Mayne, 1984; 4. Toole and Trelstad, 1971; Hay, 1980; 5. von der Mark et al, 1977; Linsenmayer, Gibney and Little, 1982; 6. Hayashi et al, 1988.

The DNA content was also measured and this levelled off after day 13, indicating that although the cell number was not increasing, the secretory capacity of the fibroblast was increasing. This is in accord with Hay and Revel (1969) who observed that the number of lamellae (i.e. cells) remains constant, while the thickness of the stroma continues to increase (after compaction), indicating accumulation of extracellular material.

Type V collagen was not detected by immunofluorescence until 6 days of development (Linsenmayer, Fitch and Mayne, 1984), suggesting that it is synthesized by the invading mesenchyme. In the chick, type II collagen is restricted to the anterior stroma, in the region which becomes Bowman's layer (von der Mark et al, 1977). Type II collagen accounts for 30-46% of the total collagen in 6-7 day embryos (Linsenmayer, Gibney and Little, 1982). In situ hybridization demonstrated type I and type II mRNAs in the epithelium at stage 18, increasing between stages 26-31 and decreasing to background levels by stage 39 (day 13) (Hayashi et al, 1988). A small amount of mRNA was present in the endothelium throughout these stages. At stage 31 type II collagen was seen in the sub-epithelial and sub-endothelial regions and in the anterior stroma, and by stage 39 it was confined to the sub-endothelial region and the anterior stroma, and had diminished by stage 43 (Hayashi et al, 1988). It was impossible to say how much type II collagen was present at these stages, as this study was not quantitative.

After stage 30, type III collagen is lost from the chick cornea (Trelstad, Hayashi and Toole, 1974; von der Mark et al, 1977) and could not be detected in 17 day cornea by immunofluorescence (Conrad, Dessau and von der Mark, 1980). Type VI collagen has been demonstrated in 19 day cornea by immunohistochemistry (Linsenmayer et al, 1986). Type VI appears to be a major component of the human cornea (Zimmermann et al, 1986), but was not detected in cultures of human stromal fibroblasts (Stoesser, Church

and Brown, 1978). Type VI seems to be located between the fibrils, as immunolocalization studies showed 6-10 nm filaments extending from the type I fibrils (Alper and Amenta, 1986). Type III was demonstrated in human stromal fibroblasts (Yue, Baum and Smith, 1983). Type IV collagen may be present as a component of the epithelial basement membrane (Trelstad, Hayashi and Toole, 1974) and in Descemet's membrane. Type VII collagen was demonstrated in human, primate and rabbit cornea (Gomez, Smith and Jester, 1986). In bovine cornea, type III may be present (Schmut, 1978). A uniform distribution of type II collagen has been demonstrated in mouse cornea (Harnisch et al, 1978). Descemet's membrane contains both type IV and type VIII collagen (Labermeier and Kenney, 1983). Type XII collagen mRNA was detected in 6 day cornea (Gordon, Gerecke and Olsen, 1987).

5.3.2 Glycosaminoglycans in the Cornea

Glycosaminoglycans constitute 0.2-1% of the wet weight of the cornea (Parry and Craig, 1984). Like all other sulphated GAGs, KS is attached to a protein core, but whereas skeletal KS is O-linked to ser or thr, corneal KS is N-linked to asn (Scott and Haigh, 1988). Corneal KSPG contains only 1-2 GAG chains in a PG of 1 x 10^5 daltons (Gregory, Cöster and Damle, 1982). The chondroitin sulphate of cornea contains iduronic acid as the uronic acid component and therefore qualifies as a CS-DS PG (Stuhlsatz, Muthiah and Greiling, 1972).

A comprehensive study of GAGs in the epithelium, endothelium and stroma has been carried out (Hart, 1978). Most of the keratan sulphate was synthesized by the stroma. After 17 days, corneal heparan sulphates (HS) appeared to be derived predominantly from epithelium. The endothelium produced large amounts of hyaluronic acid. As development proceeds the proportion of HS relative to other GAG declined. The 5-day epithelium produced CS and

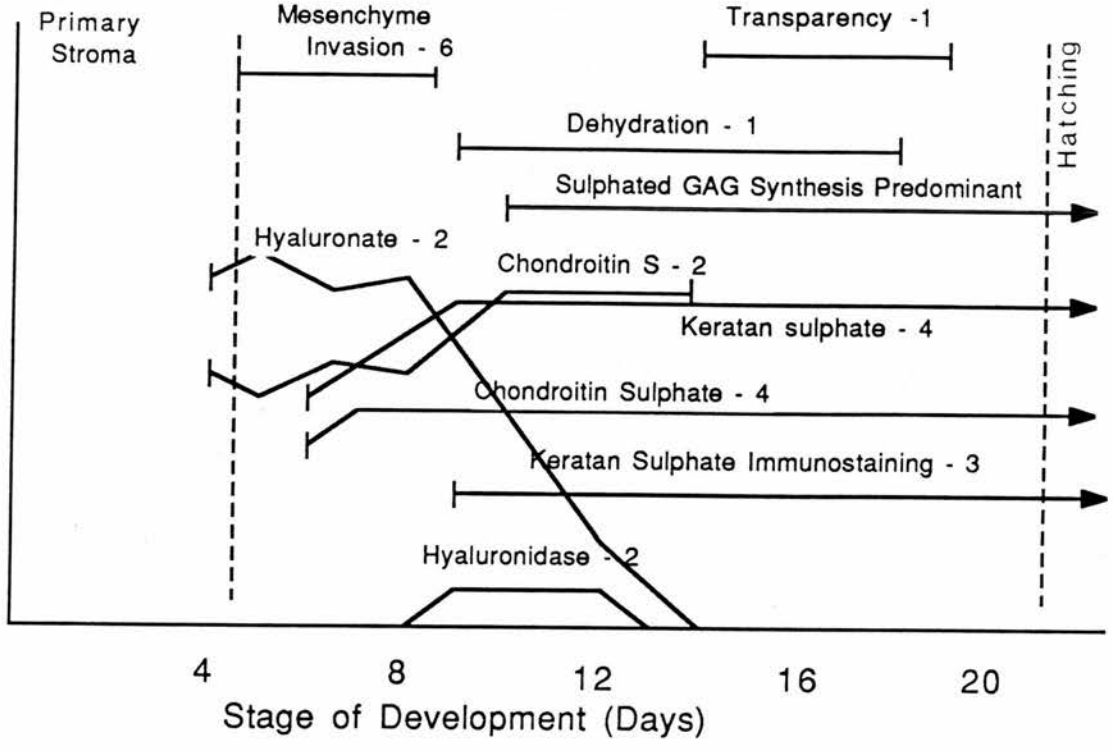

Figure 5.3 Diagramm atic representation of the changes that occur in the synthesis of proteoglycans and glycosaminoglycans types during the development of the chick cornea. The x axis shows the days of development. The changes in synthesis of the GAGs and PGs are shown in relation to the major events in corneal morphogenesis: the formation of the primary stroma, mesenchyme invasion and the development of dehydration and transparency.
1. Coulombre and Coulombre, 1958; 2. Toole and Trelstad, 1971; 3. Funderburgh, Caterson and Conrad, 1986; 4. Hart, 1976; 5. Hay, 1980.

HS in equal amounts, but at 6 days CS was the principal component and then declines to about 50% of the total epithelial GAG at 12 days (Meier and Hay, 1973). Chondroitin sulphate was present in small amounts by day 6 (stage 29) and this greatly increased by day 7, levelling off between days 7 and 14 (Hart, 1976). A peak in the ratio of chondroitin-4-sulphate to chondroitin-6-sulphate occurred at day 14, coincident with the onset of transparency and dehydration. KS was synthesized in small but significant amounts by day 6, and approached adult levels and plateaued at day 14 (Figure 5.3). Hart (1976) found no correlation between the onset of transparency and KS biosynthesis although a correlation existed between the onset of transparency and dehydration and the degree of sulphation of KS. KSPG was detected by immunofluorescence from stage 29, but extracellular staining did not occur until day 9, and occurred in a posterior to anterior direction in the stroma, simultaneously with the flattening and layering of stromal cells and coincident with the order of progression of the invading stromal cells. An exponential increase in KSPG occurred five days before hatching (Funderburgh, Caterson and Conrad, 1986). The accumulation of KSPG in the stromal cells is coincident with the increase in the rate of incorporation of radioactive precursors during the same time (Hart, 1976).

Hyaluronate is produced by the early cornea during swelling and subsequent invasion and this is removed by hyaluronidase (Toole and Trelstad, 1971). No hyaluronic acid is produced by the epithelium, so the endothelium must be the source of HA (Meier and Hay, 1973; Trelstad, Hayashi and Toole, 1974). These authors suggest that accumulation of HA may account for the swelling of the primary stroma and initiation of mesenchyme invasion at stage 27/28, by osmotic effects, since HA is produced at stages 24-26. Possibly the closure of the endothelium at stage 27/28 provides a semipermeable membrane which allows

the HA to exert an osmotic pressure. However, as HA is produced both before and after swelling, this is unlikely (Hay, 1980). CS seems to be the main GAG produced by the epithelium (Trelstad, Hayashi and Toole, 1974). There was a fall in the amount of GAG between the cornea and the corneolimbus which was due mainly to the fall in KS. There was more GAG in the cornea than in the limbic regions and the sclera (Borcherding et al, 1975). DS replaces KS as the dominant GAG in the sclera.

5.4 Disorders Affecting the Cornea

For a review and more references, the reader is referred to Freeman (1982a).

5.4.1 Keratoconus

In this disease thinning and dilatation of the cornea occur due to increased distensibility. It is not yet clear whether the thinning is due to a reduction in the number of lamellae, or a reduction in the number of individual lamellae. The disorder can occur sporadically or in conjunction with other connective tissue disorders, and can be a recessive or dominant form of inheritance (Freeman, 1982a).

Yue, Baum and Smith (1983) showed that in keratoconus, there was a small, but significant increase in the amount of type V collagen synthesized, and that some type III might be present. However, in a careful study, Zimmermann et al (1988) did not find any difference in the amounts of the collagen types present in the cornea in cases of keratoconus.

5.4.2 Osteogenesis imperfecta

In osteogenesis imperfecta (OI), the cornea is often thinner than normal, sometimes showing keratoconus or

megalocornea (Freeman, 1982a). In one case of the lethal perinatal form of OI, the level of lysine hydroxylation increased by 30-40%, paralleling an increase in bone hydroxylation (Freeman, 1982a).

5.4.3 Ehlers-Danlos Syndrome VI

Ehlers-Danlos Syndrome VI (EDS VI) is known as the ocular form, because of its association with severe ocular problems. This form of EDS is associated with a reduced activity of lysyl hydroxylase (Freeman, 1982a).

5.4.4 Keratoglobus

In this disease the cornea is transparent, but shows extreme thinning of the stroma. There appear to be no differences in the ratios of collagen types (Freeman, 1982a), or in the levels of lys hydroxylation, but there are differences in the level of glycosylation and the ratio of di:mono glycosides.

5.4.5 Sclerocornea

Here the cornea is opaque, with collagen fibrils of increased and more variable diameter (for references, see Harding, Crabbe and Panjwani, 1980).

5.4.6 Macular corneal dystrophy

In this disorder, opacity occurs and there is a deficiency in synthesis of KSPG (Poole, 1986). There seems to be a lack of sulphation of the KS chains (Poole, 1986).

5.4.7 Corneal scarring

When rabbit corneas are wounded, opaque scars form which become progressively less opaque, approaching the normal

levels of transparency 1.5 years later (Cintron and Kublin, 1977). The healing wounds showed a foetal pattern of GAG synthesis, with the low-sulphate form of KS, HA and HS present (Cintron and Kublin, 1977). The stromal lamellae varied widely in thickness and in the size of the fibrils, with a range of diameters of 5-50 nm (Cintron et al, 1978).

Thus, the defects which could affect corneal fibrillogenesis are: OI (where lys hydroxylation is high), EDS VI (decreased lys hydroxylation) and keratoglobus, possibly because the level of post-translational glycosylation will be affected, which has been proposed to affect the final fibril diameter (see Section 1.6.4.2), and possibly macular corneal dystrophy, if there is a defect in the KS as this is another factor proposed to regulate fibril diameter (see Section 1.6.4.1). Those conditions in which fibril diameter is known to be affected are sclerocornea and corneal scarring. As yet, no disorders involving defects in propeptide removal have been reported for the cornea.

5.5 Procollagen Processing in the Cornea

As yet, procollagen processing in whole cornea, or corneal cells or tissues, has not yet been examined. If the order of removal of the propeptides does affect the final fibril diameter, then it might be expected that the route of type I procollagen processing would be different in cornea and tendon.

After SDS-PAGE of extracts of medium from human corneal stromal cells in culture, type I pN-collagen was identified (Stoesser, Church and Brown, 1978). However, this was identified after long term labelling of the cells with ^{3}H proline, and so cannot be described as a kinetic intermediate. The presence of pN-collagen is interesting in view of the proposal that processing via pN-collagen leads to narrow fibrils and processing via pC-collagen

leads to wide fibrils.

In this study, processing of type I procollagen was examined in intact corneas at 12, 14 and 17 days of embryonic development (Hamilton, 1952), and the rate constants k_1, k_2, k_3 and k_4 were determined (Section 3.6). These rate constants were compared with those determined in tendon at the same developmental stages. Fibril diameter distribution data were obtained at the three different developmental stages (Section 3.9), in order to see if a correlation existed between the rate constants and the diameters. The pulse-chase protocol was the same as in Table 3.2. For each time point, three corneas were pooled.

5.6 Results

5.6.1 SDS-Polyacrylamide gel electrophoresis

Photographs of fluorograms of experiments at 12 and 17 days of development are shown in Figures 5.4 and 5.5 respectively.

It is apparent from the figures that the rate constants of procollagen processing in 12 day cornea apparently differ from those in 12 day tendon (Figure 4.2). This can be deduced from the observation of a pN intermediate in the 12 day cornea gel tracks (e.g. chase time t= 10), identified by a comparison with a pN standard (Figure 3.8). Bands migrating in the position of pNα1 and pNα2 were not observed in 12 day tendon. Thus, the route of processing observed by visual inspection of the fluorograms was apparently random. The pN-collagen intermediate was never completely processed at 12 days of development; about 10% remained even after 180 minutes of chase time. However, it is possible that this remaining pN would be further processed, as the amount of pN-collagen present at later chase times seemed to be declining.

There was a distinct change in the amounts of the

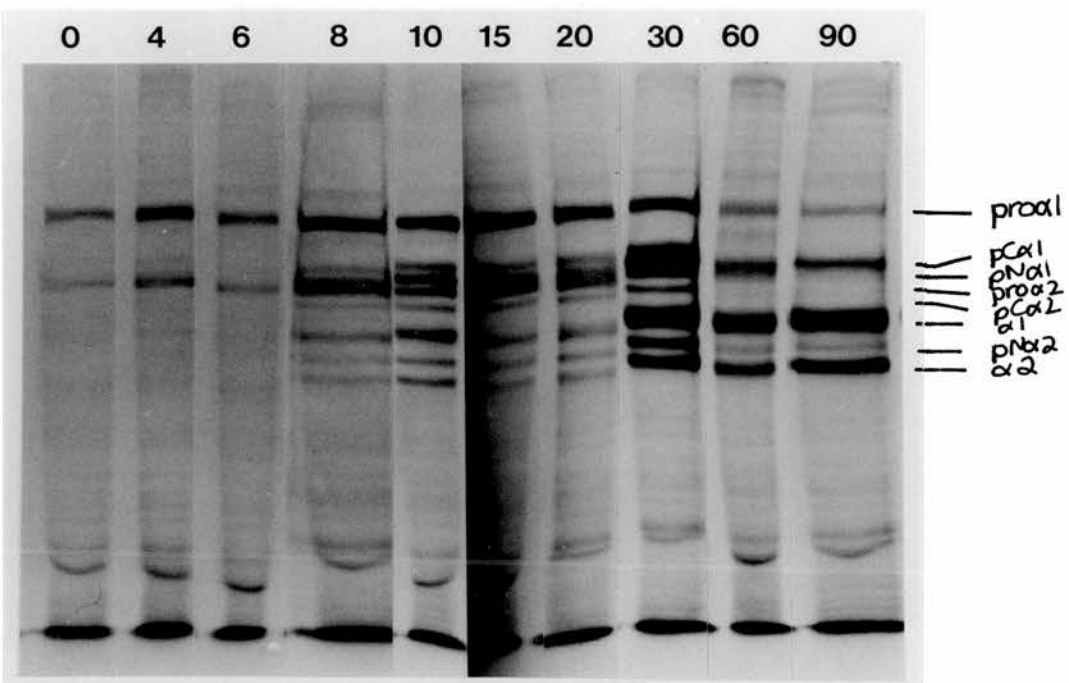

Figure 5.4 Fluorogram showing the time course of processing of type I procollagen in 12 day chick embryo corneas. Corneas were incubated with 25 μCi/ml L-5-^{3}H-proline for 20 minutes; after this time the medium containing the radioactive label was removed and replaced with 0.1 mg/ml unlabelled proline as described in Table 3.2. The tissues were extracted with a 1 M salt solution and the extracts were loaded on SDS-PAGE. The numbers above the gel tracks refer to the chase time in minutes.

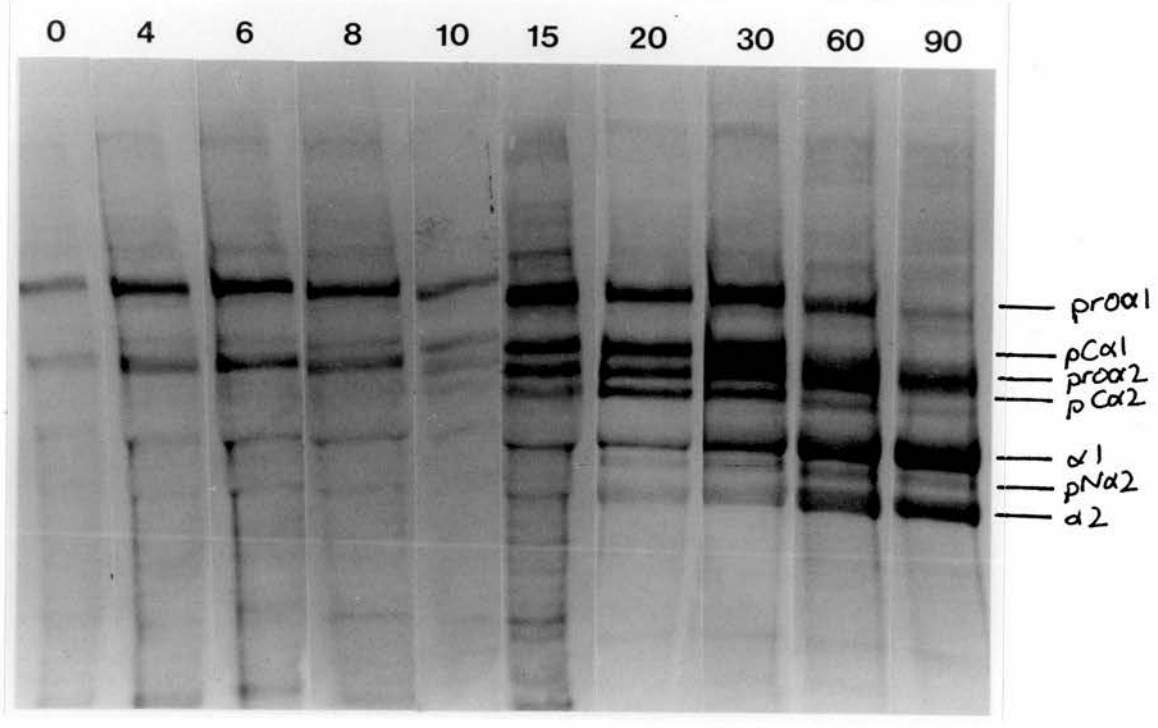

Figure 5.5 Fluorogram showing the time course of processing of type I procollagen in 17 day chick embryo corneas. Corneas were incubated with 25 μCi/ml L-5-^{3}H-proline for 20 minutes; after this time the medium containing the radioactive label was removed and replaced with 0.1 mg/ml unlabelled proline as described in Table 3.2. The tissues were extracted with a 1 M salt solution and the extracts were loaded on SDS-PAGE. The numbers above the gel tracks refer to the chase time in minutes.

various components with time (Figure 5.4 and 5.5) which could then be quantitated by densitometry.

5.6.2 Densitometry

Densitometric scans are shown for chase times t=0, 20, and 90 at 12 days (Figure 5.6) and 17 days (5.7). In most cases the bands were well resolved by the densitometer. The values of the integrals were corrected for the width of the band and the loss of ^{3}H-pro and ^{3}H-hypro due to the loss of the propeptides (Section 3.8).

Initial results with the densitometer showed that in some cases it was difficult to resolve the pCα1 and pNα1 bands or the pNα1 and proα2 bands from each other. In an attempt to improve the resolution of these bands, SDS-PAGE of the proteins extracted at various chase times was carried out at a lower acrylamide concentration, 5% acrylamide instead of 6% acrylamide. The lower percentage of acrylamide will result in a larger pore size, allowing greater separation of the chains (Section 2.3.2). Despite this, resolution of the chains was still not possible in some cases. An effort to obtain greater separation was made by continuing electrophoresis for 30 minutes after the dye front had reached 0.5 cm from the bottom of the gel.

If resolution of the bands was still not possible, values were obtained for the percentage of pCα1 and pNα1 by assuming that

$$[pro\alpha1] + [pC\alpha1] + [pN\alpha1] + [\alpha1] = 66.7\%$$

and

$$[pro\alpha2] + [pC\alpha2] + [pN\alpha2] + [\alpha2] = 33.3\%$$

and then

$$[pC\alpha1] = 2 \times [pC\alpha2]$$

and

$$[pN\alpha1] = 2 \times [pN\alpha2]$$

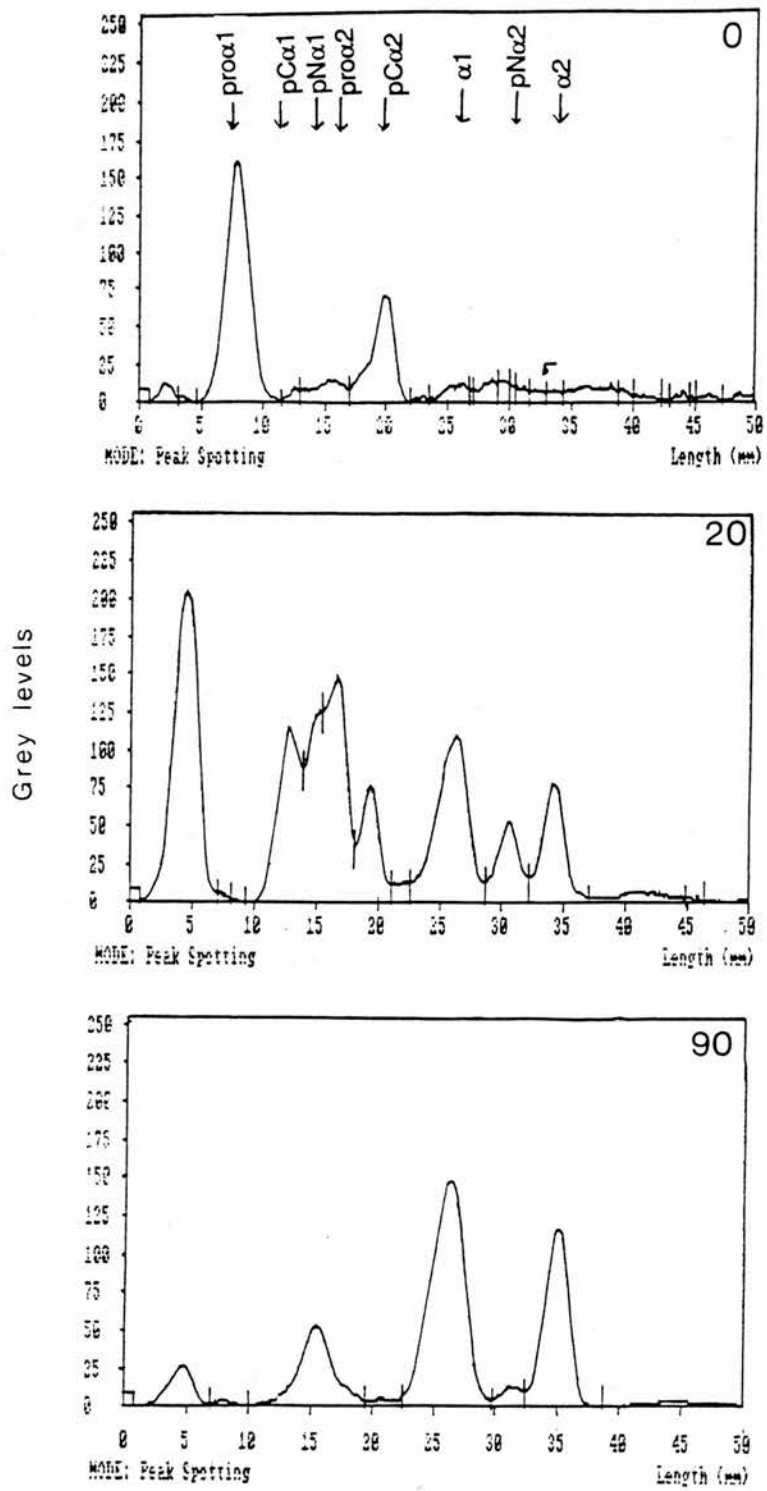

Figure 5.6 Densitometric scans of 12 day corneal fluorogram for the chase time points 0, 20 and 90 minutes in the pulse-chase experiment. The fluorograms were scanned at 530 nm.

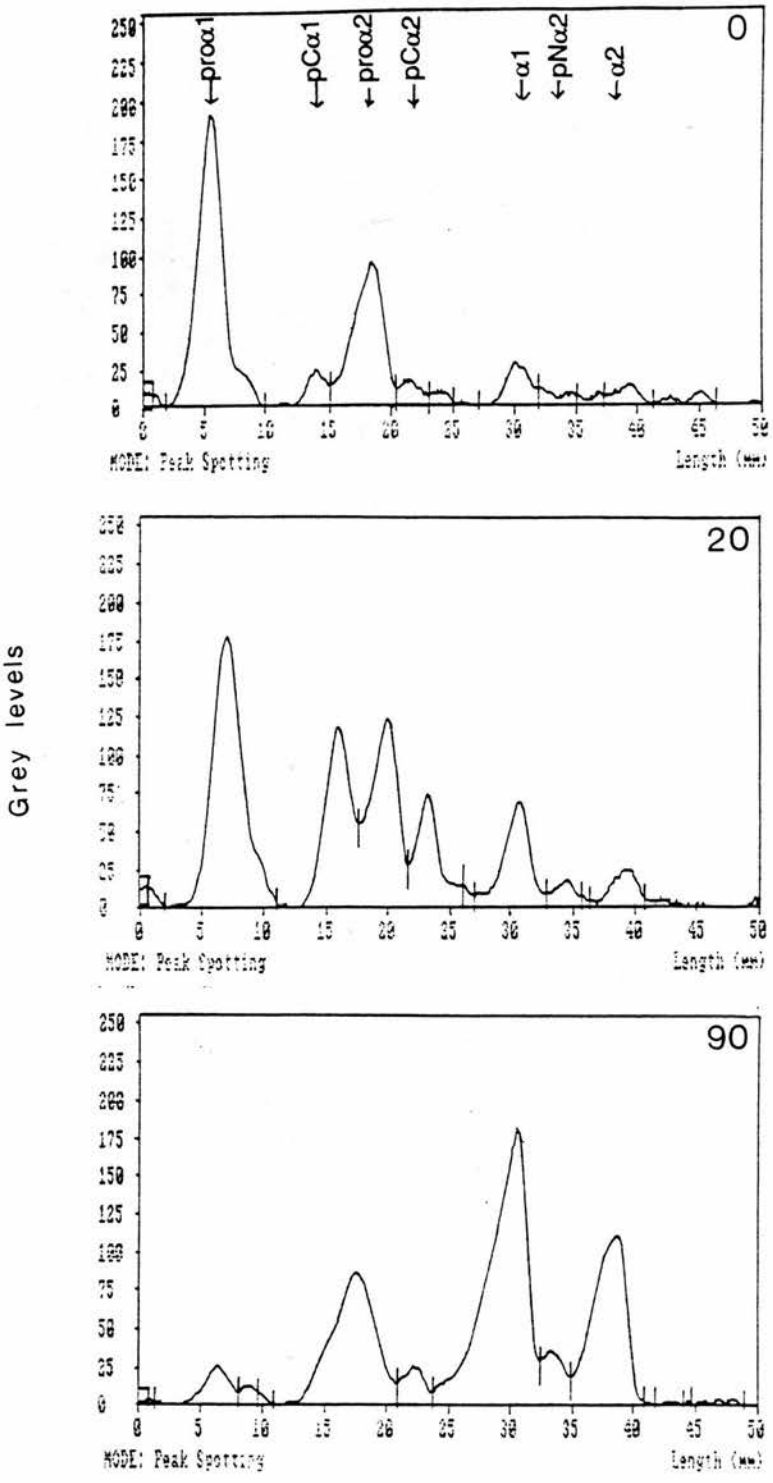

Figure 5.7 Densitometric scans of 17 day corneal fluorogram for the chase time points 0, 20 and 90 minutes in the pulse-chase experiment. The fluorograms were scanned at 530 nm.

5.6.3 Rate constants

The values obtained for k_1, k_2, k_3, and k_4 (Figure 3.1) are shown in Table 5.2. The results are apparently different from tendon, with k_4 being less than k_3; in tendon, k_3 was less than k_4.

To ascertain that these values of the rate constants corresponded to the true minimum of the contour map (i.e. the lowest value of the sum of the squares of residuals), all possible minima were determined over a range of values of initial estimates ranging from 0.0001 to 1 (a 10,000 fold range). Local minima were determined over this range. The values of the rate constants at these minima were then used as initial estimates in the minimization program. The initial estimates resulted in the values given in Table 5.2, with only two exceptions. The two exceptions resulted in values of the SSQ greater than the min SSQ of the values in Table 5.2. Hence, these exceptions were not the true values of the rate constants and they were discarded. Thus, as no other combinations of rate constants producing lower values of the min SSQ were found, the values of the rate constants of Table 5.2 were concluded to be an accurate description of the system.

In some cases, the local minima found which were used as initial estimates in the minimization program did not result in values of rate constants, i.e. the program could not fit the estimates. The values of the initial estimates were scrutinized for common features which resulted in fitting.

12 Day α1-Type Chains

If k_1 was greater than k_3, or k_1 was less than k_4, a fit could not be obtained. For the actual values of the constants (Table 5.2), k_1 is less than k_3, and greater than k_4, and k_2 is greater than k_4 and less than k_3.

12 Day α2-Type Chains

The best fit values of the rate constants were all of the same order of magnitude. Combinations of the various

α1-type chains

	12 days	14 days	17 days
k_1	0.0316 +/- 0.00274	0.0265 +/- 0.00752	0.0247 +/ 0.00246
k_2	0.0186 +/- 0.0026	0.0159 +/- 0.00635	0.0136 +/- 0.00163
k_3	0.106 +/- 0.0144	0.0985 +/- 0.04	0.0527 +/- 0.014
k_4	0.00777 +/- 0.00205	0.0067 +/- 0.00726	0.00625 +/- 0.00075
min SSQ	174.8	3607.7	101.4

α2-type chains

	12 days	14 days	17 days
k_1	0.0325 +/- 0.00245	0.0367 +/- 0.00351	0.0317 +/ 0.00362
k_2	0.0198 +/- 0.0021	0.0117 +/- 0.00151	0.00837 +/- 0.00132
k_3	0.0781 +/- 0.0121	0.087 +/- 0.0083	0.0353 +/- 0.00343
k_4	0.018 +/- 0.00287	0.0075 +/- 0.00130	0.00776 +/- 0.00153
min SSQ	19.1	34.2	46.6

Table 5.3 Values of rate constants obtained by regression analysis of pulse-chase data for the cornea. The data are expressed as: rate constant (k_i) min^{-1} =/- standard deviation. The value of the minimum of the sums of squares of the residuals is given for each of the chains.

values of the global search program in the minimization program resulted in fitting if k_1 was less than, or equal to, k_3. A fit was obtained whether k_1 was less than or greater than k_4. If k_1 was greater than k_3, no fit was obtained. If the initial estimate of k_1 and k_2 was 0.381, and k_3 was 0.061, another minimum was obtained with final values of the rate constants being k_1 = 3.81; k_2 = 3.81; k_3 = 0.048; k_4 = 0.013. The value of the min SSQ was 136, whereas the min SSQ for the values quoted was an order of magnitude lower than this (Table 5.2). Hence, the alternative values for the rate constants were rejected. The standard deviation parameters for these minima were unacceptably high (> 1000% for k_1 and k_2), so this reinforced the decision to reject these values.

14 Day α1-Type Chains

In order for an initial estimate to be fitted, k_4 must be low (less than k_1; but not if k_1 is of the order of magnitude of 10^{-1}). The initial estimate of k_3 also had to be greater than k_4. No fit was obtained if k_3 was less than k_4.

14 Day α2-Type Chains

The values shown in Table 5.2 were generally obtained whichever parameters of the local minima were used as initial estimates in the minimization program. No fit was obtained if k_1 was less than k_2, or if k_1 was of the order of 10^{-1} and was much greater than k_4. When k_1 and k_3 were much lower than k_2 and k_4, an alternative set of parameters was generated, but these were discarded as the value of the sum of the squares of residuals was much higher than the best fit minimum.

17 Day α1-type chains

The value of k_4 in the initial estimate generally had to be less than k_3 for a fit to be obtained, but this was not necessarily the case.

17 Day α2-type chains

A fit was obtained when k_1 was of the same order of magnitude as k_3, and k_4 was 10-fold lower. No fit was

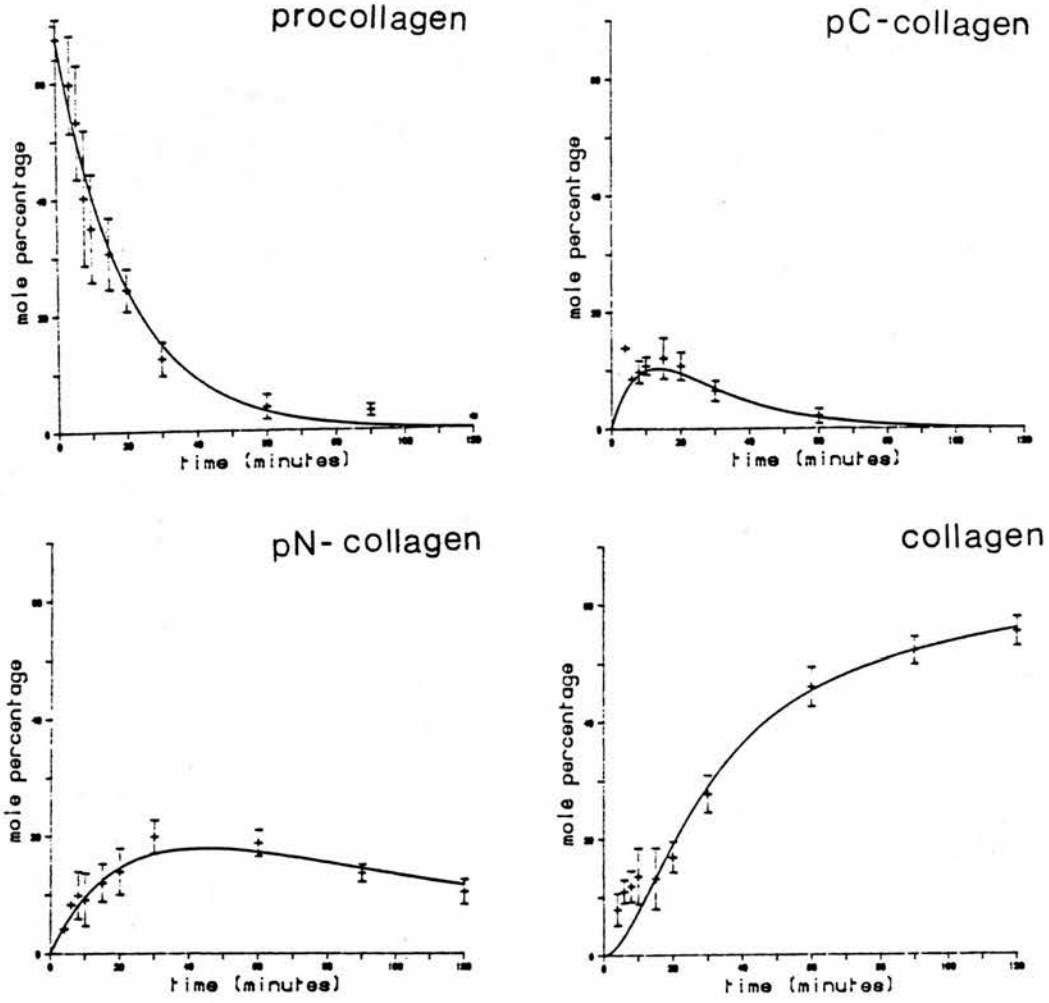

Figure 5.8a Fitted curves for the 12 day corneal data, α1-type chains. The fitted curves were obtained by using the rate constants of the pulse-chase data in the equations of Figure 3.4, calculating the concentration of each species at each time point and plotting the points obtained.

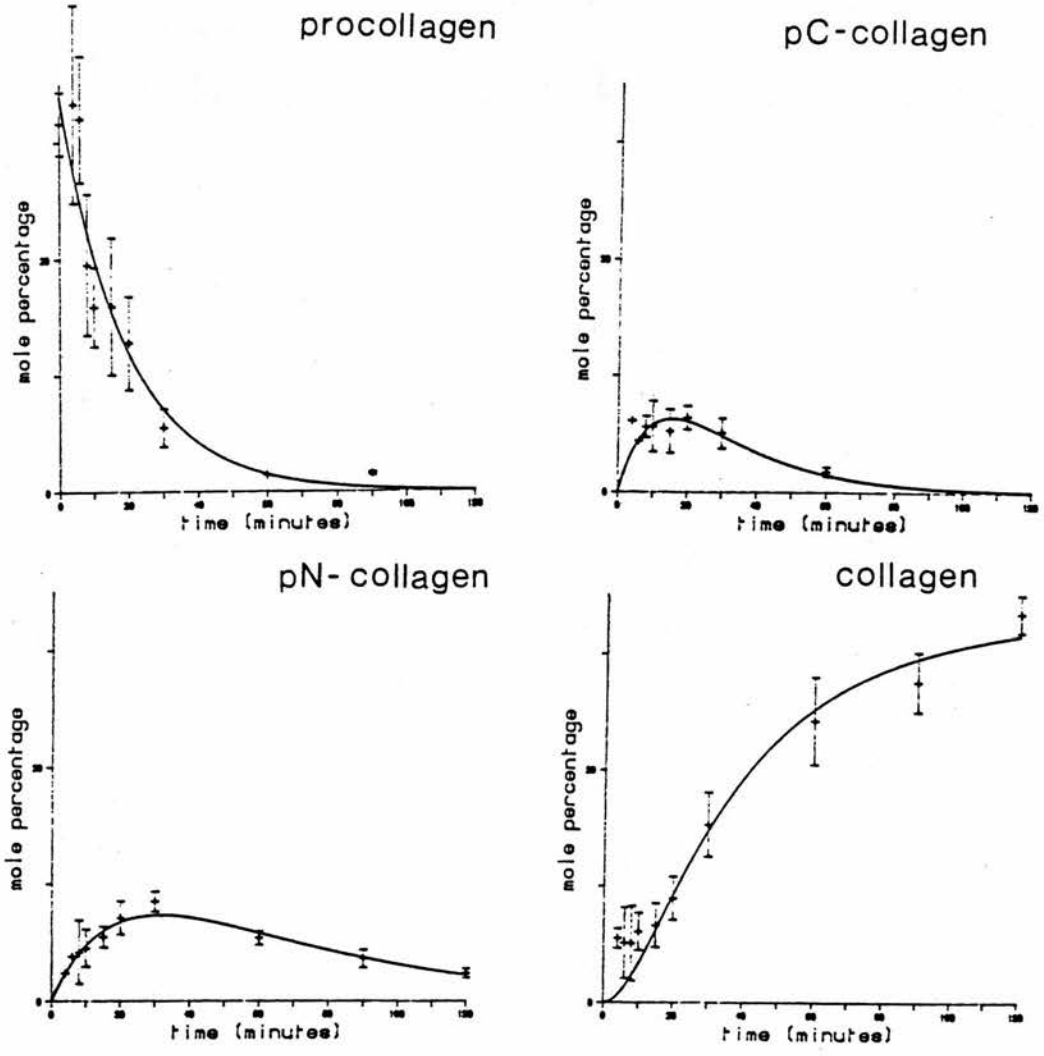

Figure 5.8b Fitted curves for the 12 day corneal data, α2-type chains. The fitted curves were obtained as described in Figure 5.8a.

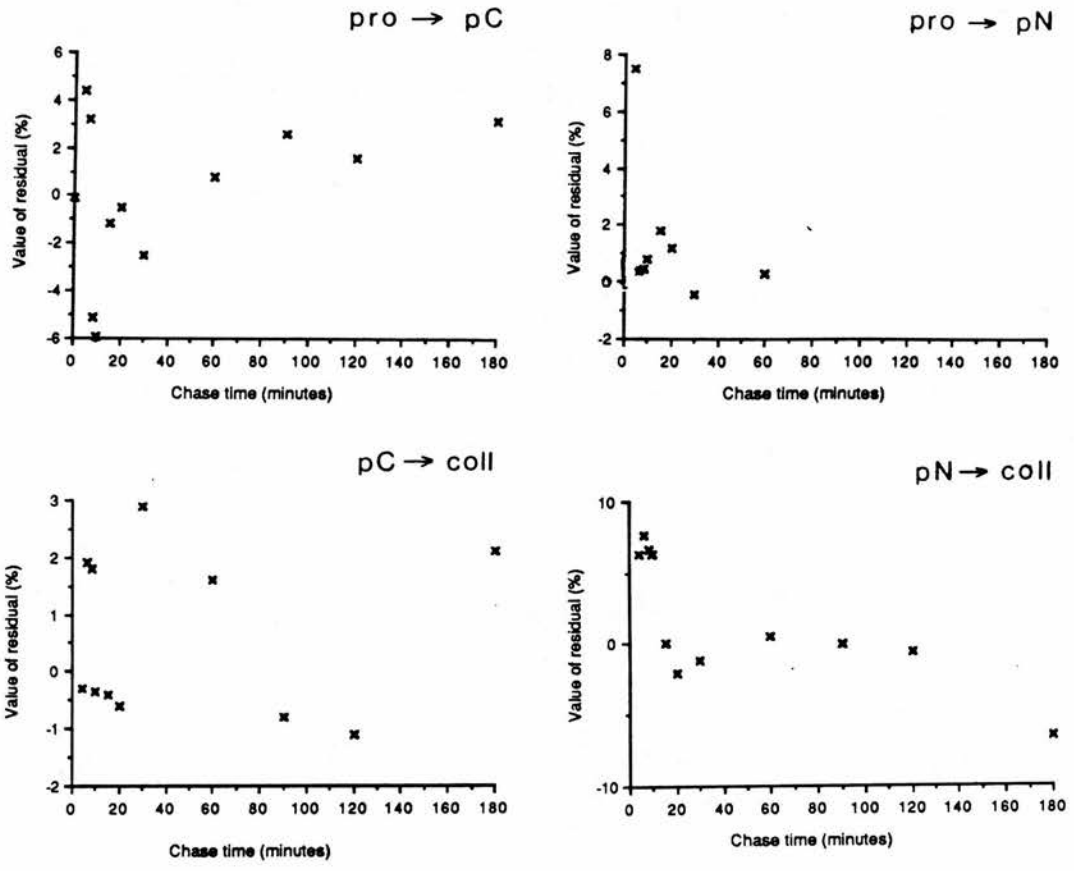

Figure 5.9 Residual plots of the 12 day cornea α1-type chains. These residual plots were selected as being typical of all the corneal data.

obtained when k_1 was less than k_4, or when the initial estimate of k_1 was of the order of 10^{-1}.

Thus, in general, a fit was obtained when the initial estimate of k_4 was less than k_3 and/or k_1. This reflects the final values of the parameters, with k_4 being less than k_3 and k_1.

5.6.4 Validity of the curve fitting techniques

The fitted curves for the 12 day chains are shown in Figures 5.8a and 5.8b. In general the fit of the curves to the data is good. The standard deviation parameters of the corneal rate constants are lower than the values of the tendon rate constants, with most of the standard deviations of the rate constants being less than 20%. The residual plots for the 12 day α1-type chains are shown (Figure 5.9) as being representative of the data. As in tendon (Figure 4.8), there is some clustering of positive and negative residuals, with the change of sign being less than 30% of the total number of changes possible.

5.6.5 Electron microscopy

The distributions of the fibril diameters in 12 day, 14 day and 17 day cornea was measured using the Magiscan 2 (Joyce-Loebl) as described in Section 2, with the criteria of Parry, Barnes and Craig (1978). A print of a micrograph from 17 day cornea which was used for determination of the diameter distribution is shown in Figure 5.10. The results are shown in Figure 5.11 in the form of histograms. It can be seen that the distribution of the diameters is sharp and unimodal, and that the mean diameter remains constant at 20-22 nm throughout the stages of development studied. This can be compared with the situation in adult cornea where the diameter remains constant at 24 nm throughout adult life (Craig and Parry, 1981). Craig and Parry (1981) only measured 17-day chick fibril diameters and obtained a

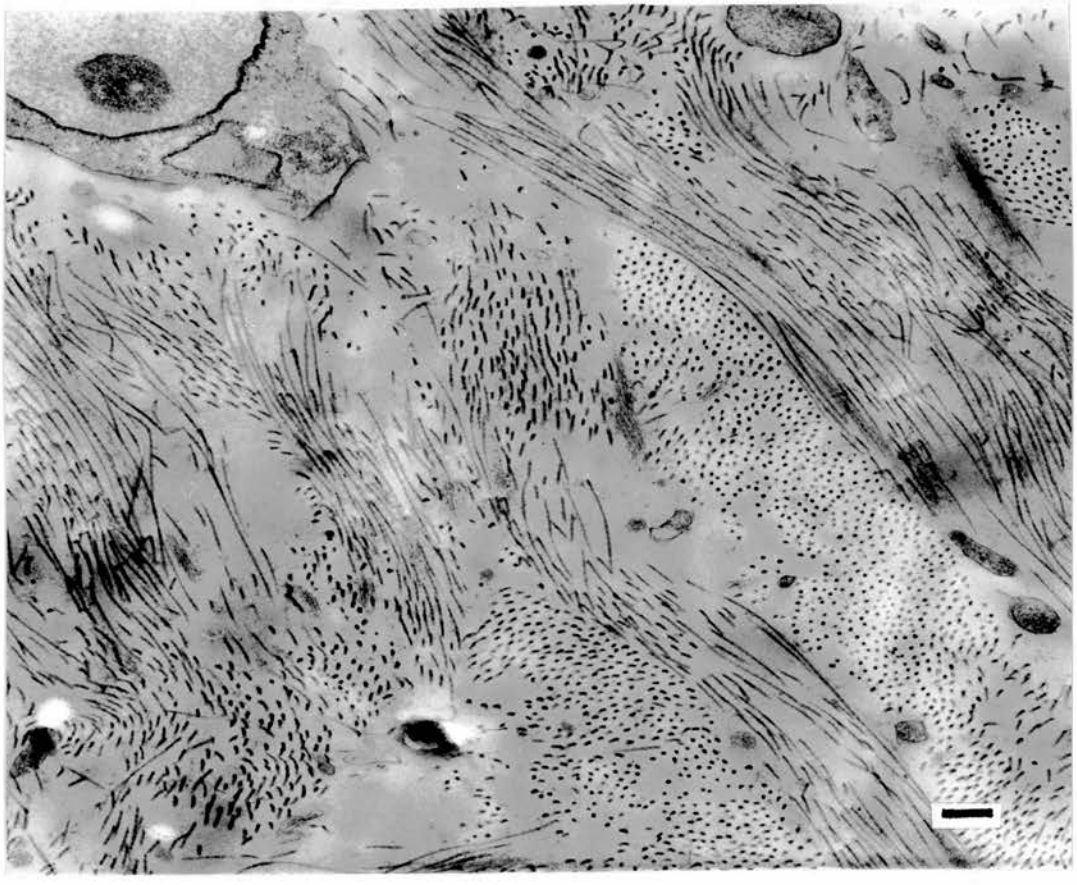

Figure 5.10 A section of 17 day embryonic chick cornea, cut perpendicular to the front surface. The tissues were stained with 1% PTA and 1% UA, dehydrated in a graded series of ethanol solutions, embedded in Araldite, sectioned and then viewed with the JEOL 1200EX electron microscope. Note the short range order of orthogonality. Courtesy Mrs C Cummmings. Magnification, x 20,0000. Bar, 300 nm. (88E/1147).

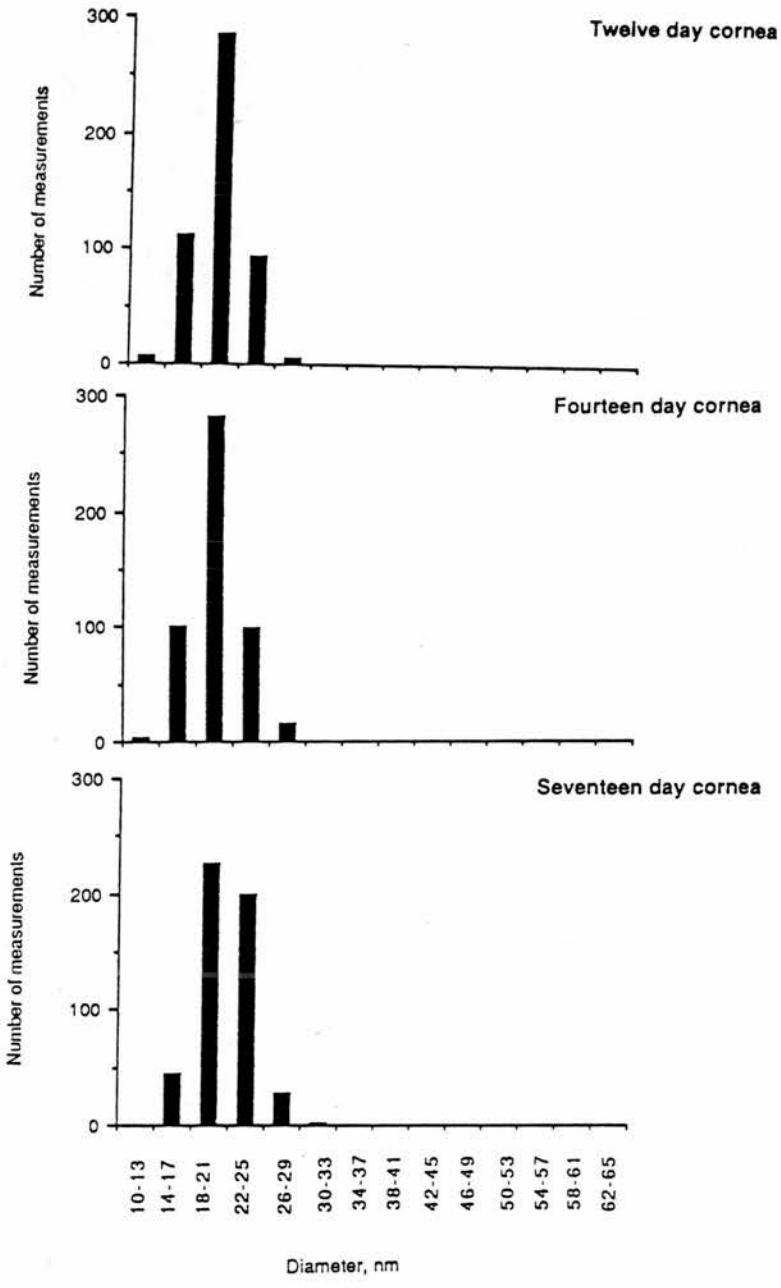

Figure 5.11 Histograms showing the distribution of fibril diameters in the corneas of 12, 14, and 17 day chick embryos. The diameters were measured using the Magiscan 3 on random areas of different sections. Five hundred measurements were taken for each developmental stage. The diameters are shown on the same scale as the tendon measurements (Figure 4.10) so that the difference in mean fibril diameter and the range of diameters can be directly compared. Note the narrow range of diameters.

figure of 17 nm, which was consistent with the mean diameters of other embryonic tissues. Although they did not measure diameters at all stages of development in the chicken, in other species it was found that foetal corneas had diameters of 17 nm at all stages of development. Hence, if the situation in chick is analogous to other species, then the current results are consistent with the data of Craig and Parry (1981). The difference in the value of the mean diameter could result from differences in the degree of dehydration of the current samples and the samples of Craig and Parry (1981) during preparation for electron microscopy.

Fibrils at 14 days of embryonic development located in cytoplasmic recesses had diameters of about 25 nm (Birk and Trelstad, 1984). However, the number of samples used in that study was probably small, so an accurate estimate of the mean diameter was not obtained.

In conclusion, then, the fibrils of the corneas at all the ages studied had a constant diameter, whereas in tendon there was an increase only between 12 and 14 days of embryonic development.

5.6.6 Efficiency of extraction

The efficiency of extraction of the collagenous molecules from the tissues by the 1 M salt solution was determined as in Section 3.7, by separation of the ^{3}H-pro and ^{3}H-hypro present after hydrolysis on a column of Dowex AG50W-X8. The data are shown in Table 5.3. At 0, 30 and 180 minutes of chase time for the 12 day and 17 day corneas, most of the radioactivity was found in the extracts, indicating that the procollagen, processing intermediates and collagen had been extracted by the 1 M salt solution. Figure 5.12 shows the elution profile of ^{3}H-pro and ^{3}H-hypro for a chase time of 180 minutes at 17 days of development. Hopefully, the fact that most of the radioactive proteins had been extracted by the 1M salt

	Efficiency of Extraction (%)
12 days	
0	89.4
	69.9
30	70.9
	74.6
180	59.2
17 days	
0	71.4
	59.0
30	70.4
	27.8
180	82.5
	67.2

Table 5.3 Efficiency of extraction of ^{3}H-containing proteins from corneas with 1 M salt at 12- and 17-days of embryonic development, determined by chromatography on Dowex AG50W-X8 of acid hydrolyzates of extracts and tissues. The percentage extraction was determined for two samples at each chase time point.

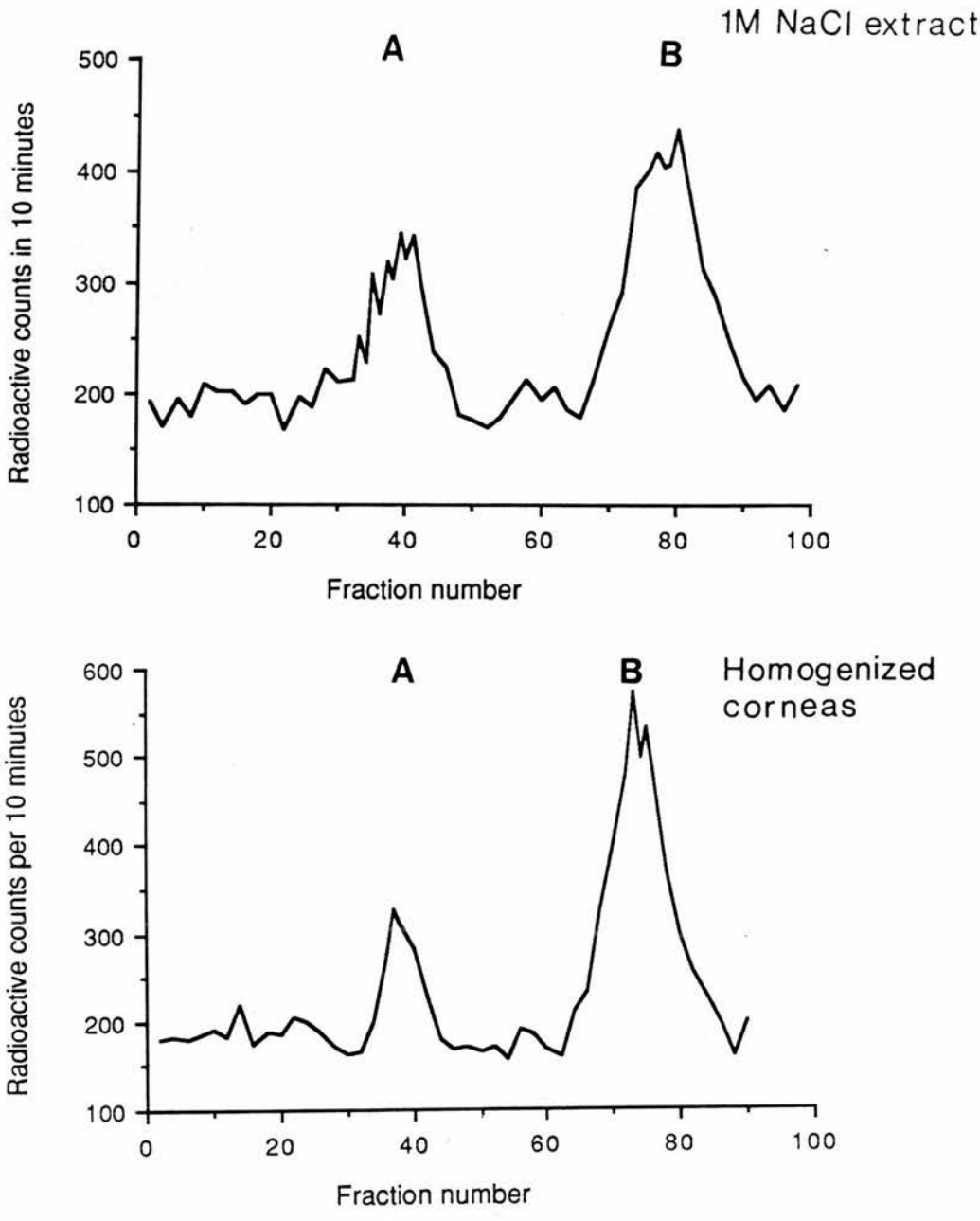

Figure 5.12 Elution profiles showing the separation on Dowex AG50W-X8 of hydrolyzed 1 M NaCl extract of 17 day corneas at 180 minutes of chase time (top) and the hydrolyzed homogenized corneas. The fraction volume was 2 ml and the flow rate was 120 ml/hr. Peak A is ^{3}H-hypro and peak B is ^{3}H-pro.

indicated that each species was extracted to the same extent.

5.7 Discussion

5.7.1 Rate constants

The change of the values of the rate constants with days of development is shown in Figure 5.13 for the α1-type chains and Figure 5.14 for the α2-type chains. For the α1-type chains, it can be seen that the values of k_1 and k_2 decrease slightly between 12 days and 17 days, and k_4 does not change significantly over the stages of development studied. For the α2-type chains, k_1 does not change during the stages of development studied. The value of k_2 decreases significantly between 12 and 14 days, and k_3 decreases between 14 and 17 days. The value of k_4 decreases between 12 and 14 days of development. All the values of the rate constants are of the same order of magnitude, except for k_4 which is less than k_3. The reason for the decrease in the values of the rate constants is not clear. A possible suggestion is that the decrease in stromal thickness which is occurring over this time (Figures 5.2, 5.3; Coulombre and Coulombre, 1958; Trelstad and Coulombre, 1971) may result in ECM molecules such as PG or type V collagen interacting with the enzymes and affecting their activity by steric hindrance due to the increased proximity of the enzyme and cofactor. It can be seen from Figure 5.3 that at the stages studied the amount of hyaluronic acid present has decreased to zero by day 15 (Toole and Trelstad, 1971). Thus, it is possible that the enzymes interact with hyaluronic acid, such that loss of HA between days 12 and 17 decreases the activity of the enzyme. Type II collagen present in the stroma is also decreasing over this time period (Figure 5.2; Hayashi *et al*, 1988).

The values obtained by the curve fitting program are

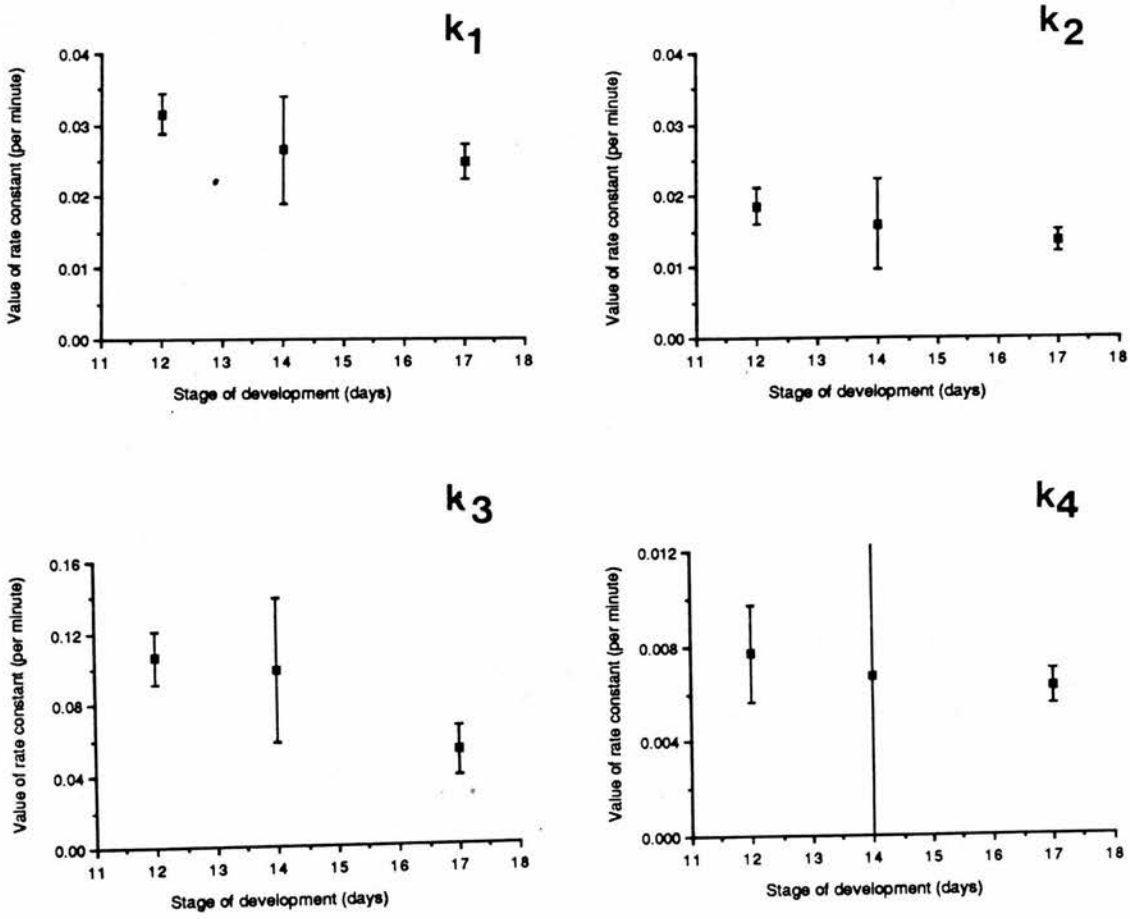

Figure 5.13 Change of the values of the rate constants with days of development for the corneal α1-type chains.

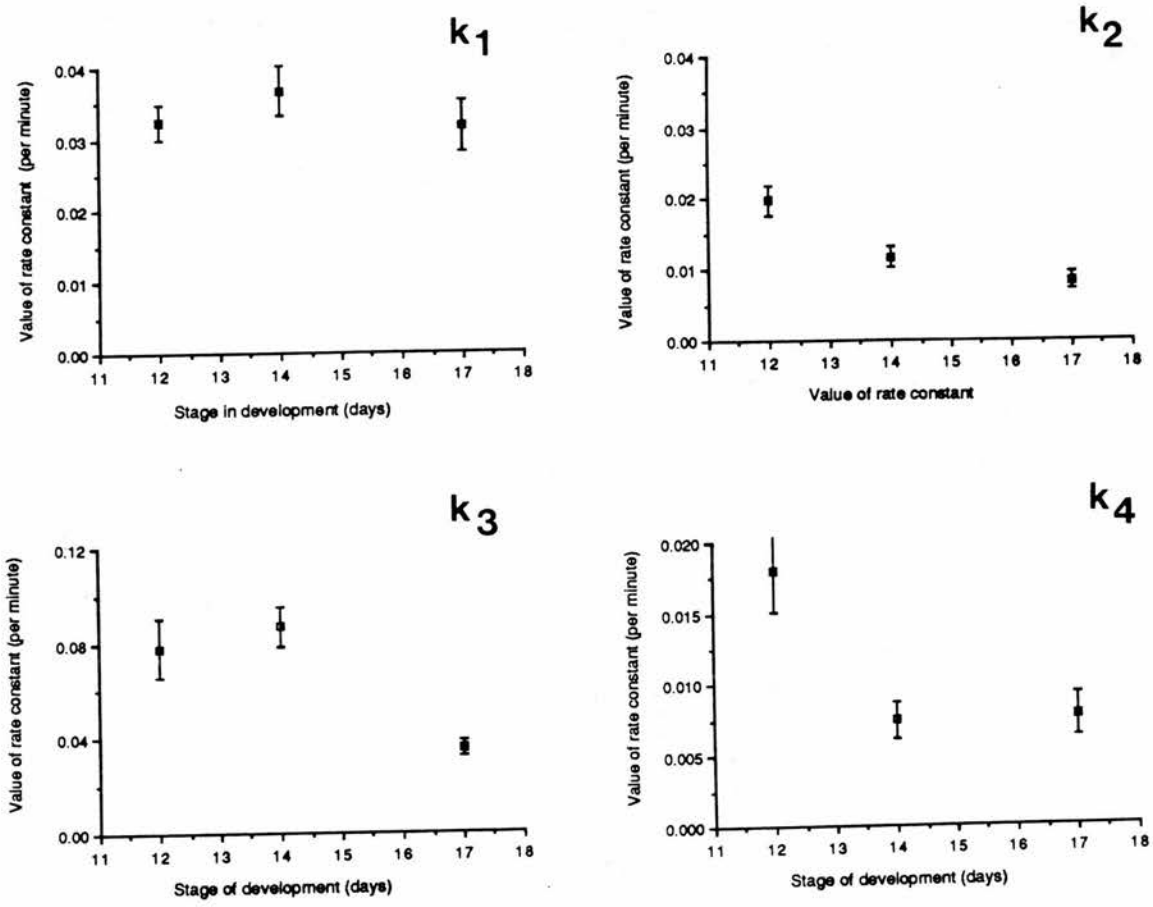

Figure 5.14 Graphs showing the change of the values of the rate constants with days of development for the corneal α2-type chains.

rate constants, not rates (Section 4.7.2), and the rate can be related to the rate constant by the expression given in Section 4.7.2. In the present experiments the concentrations of procollagen, the intermediates and collagen were not determined. Previous workers have established that from 13-20 days of embryonic development, when collagen was accumulating most rapidly, the incorporation of ^{14}C-glycine was greatest (Coleman, Herrmann and Bess, 1965). This accumulation was concomitant with an increase in the level of collagenous proteins from one-third that of the non-collagen protein at 9 days to nearly three times the level of non-collagenous protein at 20 days. Incorporation of ^{14}C-L-glycine into collagen and non-collagen protein confirmed that the uptake of the label into collagen increased during this period. Determination of the amount of DNA showed that the amount of DNA increased to day 13, but was constant thereafter (Figure 5.2). Expression of the incorporation of ^{14}C-L-gly into collagen and non-collagen protein as per μg DNA showed that the rate of incorporation into collagen increased 7-fold between days 9-16, whereas the non-collagenous protein incorporation stayed relatively constant (Coleman, Herrmann and Bess, 1965).

Hence, during the developmental stages studied in the cornea, the rate of collagen synthesis is increasing. Thus, as the rate constants k_1 and k_2 decrease slightly during this period, if the precursor concentration is increasing and the amount of collagen being laid down is also increasing, then the rate of processing may either be be increasing, remaining constant or decreasing. Even if the rate constants k_1 and k_2 are decreasing, this may not necessarily mean that the rate of processing is decreasing, as if the precursor concentration is increasing, this may overcome the effect of the decreasing value of the rate constant. This observation is interesting in view of the fact that the activities of

prolyl hydroxylase, procollagen peptidase and lysyl oxidase activity are all maximal at the same stage in cell culture (Layman, Narayanan and Martin, 1972; Layman and Ross, 1973; Cardinale and Udenfriend, 1974).

As in tendon, the value of k_1 is not the same as k_4 and k_2 is not the same as k_3, even though the enzymatic steps are the same; k_1 and k_4 are reactions involving the removal of the N-propeptide and k_2 and k_3 are the rate constants for the removal of the C-propeptide. It is not clear why the rate constants are so different even though the same reaction is being catalyzed. Removal of the C-propeptide decreases the N-proteinase activity ($k_4 < k_1$) and removal of the N-propeptide increases the C-proteinase activity ($k_3 > k_2$). This is the reverse situation from tendon.

5.7.2 Presence or otherwise of other collagen types

The presence of type V collagen in embryonic chick cornea has been noted by Kao, Mai and Chou (1982) and Pöschl and von der Mark (1980). Extraction of 17 day embryonic chick corneas which had been labelled with ^{14}C-pro (1 μCi/ml) for 24 hr with 0.5 N acetic acid and pepsin at 4°C, followed by differential salt fractionation revealed that type I collagen precipitated at 1.7 M NaCl and 2.5 M NaCl, with type V collagen precipitating at 5 M NaCl and contributing 5% of the total collagen (Kao, Mai and Chou, 1982). Matrix-free cells isolated from 17 day chick cornea and labelled with ^{14}C-pro for 2 hours indicate a value of 4% for the amount of type V (Kao, Mai and Chou, 1982). In contrast, Pöschl and von der Mark (1980) using pepsin-acetic acid extraction of 14 day corneas obtained a figure of 20% for the amount of type V. A figure of 5% type V was obtained by Welsh et al (1980) and Freeman (1978), but this was in mature rabbit cornea, not in embryonic chick.

Type V collagen first appears in the cornea at 6 days of

development and is present throughout the stroma at day 9 (Linsenmayer, Fitch and Mayne, 1984). An elegant immunogold study has shown that fibrils in the corneal stroma are heteropolymeric, consisting both of type I and type V collagen (Birk et al, 1988). The species of type V collagen present in cornea is AB_2 ($[\alpha 1]_2\alpha 2$) (Freeman, 1982a).

Thus, the presence of comparatively large amounts of type V collagen in the stroma at the stages shown, and the knowledge that types I and V occur in the same fibril, has implications for the current data. It would be expected that up to 20% of the collagen extracted by 1 M salt would be type V, and that the presence of type V collagenous bands might interfere with the quantitation of the data.

The synthesis time of a proα(V) chain is about 10 minutes (Fessler and Fessler, 1987) and tyrosine sulphation of the N-propeptide occurred 50 minutes later followed by secretion (Fessler et al, 1986). Thus, no proα(V) or pα(V) would be visible on gels until 60 minutes of chase time in the current experiments.

Processing of type V appears to take place with different kinetics from type I procollagen. Conversion of the procollagen (V) to p-collagen (V) with removal of the C-propeptide seems to occur comparatively quickly, but removal of the p-domain appears much slower (Fessler et al, 1981). Without a detailed kinetic analysis it is not possible to comment on the rates of processing, but it seems clear that the p-collagen (V) intermediate persists even 24 hours after the initiation of labelling. Sulphated proα1(V) is cleaved faster than sulphated proα1'(V) chains (Fessler et al, 1986). The apparent interval between sulphation and cleavage of proα1'(V) is 9 minutes, and 3 minutes for proα1(V) (Fessler et al, 1986). Thus, in the present system proα1'(V) and proα1(V) chains should be visible from 60 minutes of chase time onwards. Thus, the fluorograms were examined for the presence of any bands which might correspond to proα1(V), proα2(V), pα1(V) or

pα2(V). Comparison of chick type V markers prepared from blood vessels shown in the paper of Fessler, Robinson and Fessler (1981) reveals that on 4% acrylamide gels all of the chick type V chains migrate more slowly than chick proα1(I) and proα2(I), except for the α2(V) chain which migrates between the proα1(V) and the proα2(I) chain. In contrast, pepsin-extracted α1(V) and α2(V) (αB and αA) migrated only slightly more slowly than the α1(I) and the α2(I) chains (Kao, Mai and Chou, 1982) on SDS-gels of unspecified acrylamide concentration. The significance of these observations is unclear, however, as the α chains of type V collagen retain 35,000-dalton non-collagenous portions and these extensions may not remain intact after pepsin treatment. Despite the discrepancy, it seems clear that in the present study the only chains seen would be the proα and pα chains, and these would migrate more slowly than the corresponding type I intermediates. No such bands were seen. The proα2(V) chain appears to have no significant N-terminal non-collagenous extension and migrates with the same electrophoretic mobility as the pα2(V) chain when both are reduced (Fessler and Fessler, 1987). Examination of the pα2(V) band under non-reducing conditions shows that the pα2(V) migrates more slowly than the proα2(V) chain, due to the attachment of the non-collagenous peptide P (Kumamoto and Fessler, 1981; Fessler et al, 1981). Thus, virtually all of the species of type V chains present in cornea would migrate more slowly than type I components, and no such bands were seen. It must be concluded, therefore, that such molecules were not extracted by 1 M salt, or were extracted in such small amounts that they were not visible on a gel. It should be possible to extract these molecules using 1 M salt (Miller and Gay, 1982; Miller and Rhodes, 1982) and type V collagens have been successfully extracted from 19-day embryonic chick crop with a 1 M sodium chloride solution (Kumamoto and Fessler, 1981); 18 day chick blood vessels and calvaria (Fessler et al, 1981); chick tendon

fibroblasts and Chinese hamster lung cells (Fessler, Robinson and Fessler, 1981). Several investigators have noted the difficulty of extracting cornea with 1 M salt (Freeman, 1978; Welsh et al, 1980). Pöschl and von der Mark (1980) state that tissue bound type V resists extraction completely with salt or acids, suggesting that the tissue form of type V collagen is highly cross-linked. However, under the conditions used in the pulse-chase experiments, this should not affect extraction, as even if fibrils had been laid down, lysyl oxidase-initiated cross-linking would not have occurred. The cross-linking inhibitor BAPN was included in these experiments to increase extractability by inhibiting cross-linking. Control samples chased for 120 minutes and 180 minutes in the absence of BAPN showed no increase in the amount of cross-linked components present. This indicates that a) either BAPN was ineffective under these conditions b) cross-linking did not occur at these chase times anyway. No type V collagen was seen after extraction of corneas with 1 M NaCl (Birk and Lande, 1981). An alternative explanation for the absence of type V procollagen and p-collagen is that the specific radioactivity of the samples was too low to be seen by fluorography.

The presence of type VI collagen in 19 day chick stroma was shown by immunofluoroescence (Linsenmayer et al, 1986). It is not known whether type VI is present at any of the developmental stages studied. Type VI represented 25% of the total collagens in the human corneal stroma, so if type VI is present in these experiments it should migrate on reducing SDS-PAGE as bands of molecular weight 260,000, 150,000 and 140,000 daltons (Linsenmayer et al, 1986; Colombatti et al, 1987; Timpl and Engel, 1987). The 260,000 dalton chain should migrate above proα1, and no such band was seen. The 150,000 and 140,000 dalton chains should migrate on SDS-PAGE in the position of the pCα2 chain (Sear, Grant and Jackson, 1981).

Type VI begins to be secreted 45 minutes after the

initiation of synthesis in chick tendon fibroblasts, but the kinetics of secretion are very slow; only 60% of the type VI was secreted after 4 hours (Colombatti and Bonaldo, 1987). Type VI collagen can be extracted with a 1 M salt solution (Linsenmayer et al, 1986).

Thus, if type VI collagen was present at the developmental stages studied, it would be seen after 30 minutes of chase time. A possible explanation for the absence of type VI could be that type VI might not be visible on the fluorograms because of the large globular domains present in the type VI molecule; hence the specific radioactivity of the ^{3}H-type VI would be quite low due to the lower amount of proline and hydroxyproline present in type VI collagen as opposed to type I collagen. Thus, type VI might not be visible under the current conditions of exposure of the gels. The slow kinetics of secretion would result in only a small proportion of the type VI being present as a percentage of the total collagens at any of the chase time points.

Type III collagen would probably not be present at any of the developmental stages studied (Figure 5.2; Trelstad, Hayashi and Toole, 1974; von der Mark et al, 1977). Epithelia from 5-6 day old embryos synthesized 40% type II collagen as a percentage of the total collagens (Linsenmayer, Smith and Hay, 1977), so it is likely that 40% type II collagen is present in the primary corneal stroma (Linsenmayer, Gibney and Little, 1982). Type II collagen is present throughout the entire stroma at stage 26 (Hayashi et al, 1988). By stage 31, type II collagen was more abundant in the anterior stroma and gradually decreased at later stages of development, until by stage 39 (13 days) type II collagen was confined to the subepithelial region. Complementary DNA (cDNA) probes for types I and type II collagen show that the epithelium and endothelium produce types I and II during the formation of the primary acellular stroma and for the next few days after the invasion of fibroblasts (Hayashi et al, 1988).

As the study of Hayashi et al (1988) was not quantitative, it is not possible to state what percentage of the total collagen can be ascribed to type II collagen at days 12, 14 and 17. However, the amount of type II collagen at these stages is probably quite small, as it seems to be confined to subepithelial and subendothelial locations. Type II collagen was shown to be present between stages 20-30 (Table 5.1) by immunofluorescence, and again only the subepithelial and subendothelial regions stained for type II at later stages (von der Mark et al, 1977). It is possible that in the present study a small amount of type II collagen was synthesized and extracted with 1 M salt.

5.7.3 Procollagen processing and relation to fibril formation

Measurement of collagen fibril diameters in the chick cornea at 12, 14 and 17 days of embryonic development showed that the mean fibril diameter remained constant throughout.

It seems clear that there is no direct correlation between the rate constants of processing and the final fibril diameter. It might be expected that corneal type I procollagen would be processed via a pN-collagen intermediate (Fleischmajer et al, 1981, 1983; Miyahara et al, 1984). According to Figure 3.1, this implies that k_1 and k_3 are very low or zero. It is clear from Table 5.2 that this is not the case, as k_1 is appproximately the same as k_2. Thus, a direct involvement of procollagen processing in the control of fibril diameter can probably be eliminated, reinforcing the data obtained in Chapter 4.

5.7.4 Differences In Processing between Tendon and Cornea

It is clear from the results that procollagen processing differs in tendon and cornea, although the same genetic type of collagen is being processed.

In tendon, k_4 was larger than k_3, whereas in cornea k_3 was larger than k_4. In cornea k_4 was less than k_1, but in tendon k_1 was less than k_4. Similarly, in cornea, k_3 was greater than k_2, but in tendon k_2 was greater than k_3. However, this last observation is open to question, as the values of k_2 and k_3 in tendon are of the same order of magnitude. In cornea, k_3 decreased with days of development. These observations about procollagen processing suggest that possibly there are tissue-specific forms of the enzymes with different catalytic properties. There are separate N-proteinases for catalyzing the removal of the N-propeptides from types I and III procollagen (Nusgens et al, 1980; Halila and Peltonen, 1984, 1986). It is not known if different enzymes catalyze C-propeptide removal from the various collagen types. The C-proteinase purified by Hojima, van der Rest and Prockop (1985) removed the C-propeptide from types I, II and III procollagen.

Other factors that may account for the differences in processing between the two tissues include the presence of tissue-specific en hancers/inhibitors of activity. A protein of molecular weight 35,000 daltons from mouse fibroblast cultures was found to enhance the activity of C-proteinase (Adar et al, 1986). This protein also enhanced the activity of C-proteinase from chick tendon fibroblasts (Hulmes and Kessler, personal communication). The identity of the corneal processing enzymes is not known. Thus, it is entirely possible that factors exist in cornea which affect the activity of the enzymes. It is also possible that the decrease in water content and thickness of the corneal stroma between days 14 and 19 in ovo (Coulombre and Coulombre, 1958) may affect the rate constants of type I procollagen processing. Other factors in cornea which may affect the processing of type I procollagen are the presence of large amounts of type V collagen (Pöschl and von der Mark, 1980) and HA (Hart, 1978).

CHAPTER 6

IN VITRO INVESTIGATION OF COLLAGEN FIBRIL FORMATION IN THE CORNEA

6.1 Introduction

Collagen fibrils in adult tendon and cornea consist mainly of type I collagen, yet the mean fibril diameter distributions in the two tissues are different (Parry, Craig and Barnes, 1978: Craig and Parry, 1981; Parry and Craig, 1984). Collagen fibrils in the adult cornea of every species so far examined have mean diameters of 24 nm (except for sealion) and the spread of diameters is very narrow. On the other hand, the spread of fibrils in adult tendon is large, and the mean diameter can be greater by a factor of 10, as in the common digital extensor tendon of horse (Parry, Craig and Barnes, 1978). Obviously, some regulatory mechanism operates to maintain the precise diameters of the fibrils of the cornea, thought to be necessary for transparency (Maurice, 1957, 1969; section 5.1).

How corneal collagen is able to form fibrils of such uniformly narrow diameters whilst tendon collagen forms fibrils of a wider diameter is not known. Several factors have been proposed to affect fibril diameter in vivo: 1) proteoglycans and glycosaminoglycans; 2) amount of hydroxylysylglycosides; 3) interactions between collagen types and 4) procollagen processing (Section 1.6.4).

The physical properties of type I collagen from cornea and other tissue sources have been compared. The patterns of positive staining of SLS aggregates of scleral type I and corneal type I collagen were very similar, suggesting that the charge distribution of the two collagens was similar (Birk and Silver, 1983). The only difference which could be demonstrated between the two collagens was the higher molecular weight of the corneal type I collagen, due to differences in the levels of post-translational glycosylation (Grant et al, 1969; Schofield, Freeman and Jackson, 1971). Tendon and corneal type I collagens contain about the same amounts of hydroxylylsyl residues, but cornea contains 19.1 sugar units/3000 amino acids

whereas tendon contains about 4.1 sugar units/3000 amino acids.

In vitro studies of collagen fibril formation from corneal type I and other type I collagens have been carried out (Birk and Lande, 1981; Birk and Silver, 1984b; Valli *et al*, 1986; Amudeswari, Liang and Chakrabarti, 1987). By raising the pH and the temperature of a cold solution of acidic collagen, corneal collagen was shown to form fibrils 6-7 times more slowly than scleral type I collagen (Birk and Lande, 1981; Birk and Silver, 1984b). The values of the activation energies for the lag and growth phases of fibrillogenesis were larger for corneal type I collagen than for scleral type I collagen (Birk and Silver, 1984b). Plots of specific turbidity ([final turbidity/concentration]) against concentration revealed that corneal collagen had a value of 2.74 for the intrinsic turbidity whereas that for scleral type I was 9.4. The intrinsic turbidity is proportional to the fibril mass per unit length (Silver and Birk, 1983), so this indicates that the scleral type I fibrils were larger than the corneal type I fibrils formed *in vitro* (Birk and Silver, 1984b). The apparent rate constants for the lag and growth phases were smaller for corneal type I then for scleral type I. Pepsinization of corneal and scleral type I collagens decreased the rate constants of the lag and growth phases for both collagens proportionately, and increased the activation energy of the the lag phase (Birk and Silver, 1984b).

A longer lag phase and slower rate of growth of *in vitro* fibril formation of bovine corneal type I collagen was also noted by Valli *et al* (1986). The bovine adult corneal collagen fibrils had maximum diameters of 60 nm whereas reconstituted tendon type I collagen fibrils reached a maximum diameter of 144 nm. Type I collagen of calf skin which was enzymatically deglycosylated had a shorter lag time and a faster growth rate of *in vitro* fibril formation than enzymatically glycosylated calf skin collagen

(Amudeswari, Liang and Chakrabarti, 1987), consistent with other reports.

Another *in vitro* system, cleaving procollagen with the purified N- and C-proteinases, has been used to investigate fibril formation (Miyahara *et al*, 1982, 1984; Kadler, Hojima and Prockop, 1987). The system of Miyahara (1982, 1984) used chick tendon type I procollagen whereas Kadler, Hojima and Prockop (1987) purified the type I collagen from human skin fibroblasts.

In this study, purified N- and C-proteinases were used to cleave the propeptides from tendon and corneal type I procollagen, so that fibril formation *de novo* of tendon and corneal type I procollagens could be compared. The glycosylation state of the two procollagens was investigated by lectin blotting.

6.2 Materials and Methods

In vitro fibrillogenesis, electron microscopy and lectin blotting were carried out as described in Chapter 2 (Sections 2.4 and 2.5).

6.3 Results

6.3.1 Purification of corneal and tendon type I procollagen

Corneal and tendon type I procollagens were purified as described in Sections 2.1 and 2.2. The corneas and tendons were removed from the same batch of chick embryos (30 dozen), and fibroblasts were isolated and incubated with ^{3}H-proline on the same day. Thus, the procollagens were purified from the same batch of chick embryos and hence are controls for each other. Matrix-free cells were prepared from the tissues by collagenase/trypsin digestion (Section 2.1). The cells were then incubated with 1 μCi/ml (37 kBq/ml) ^{3}H-proline in modified Krebs II medium

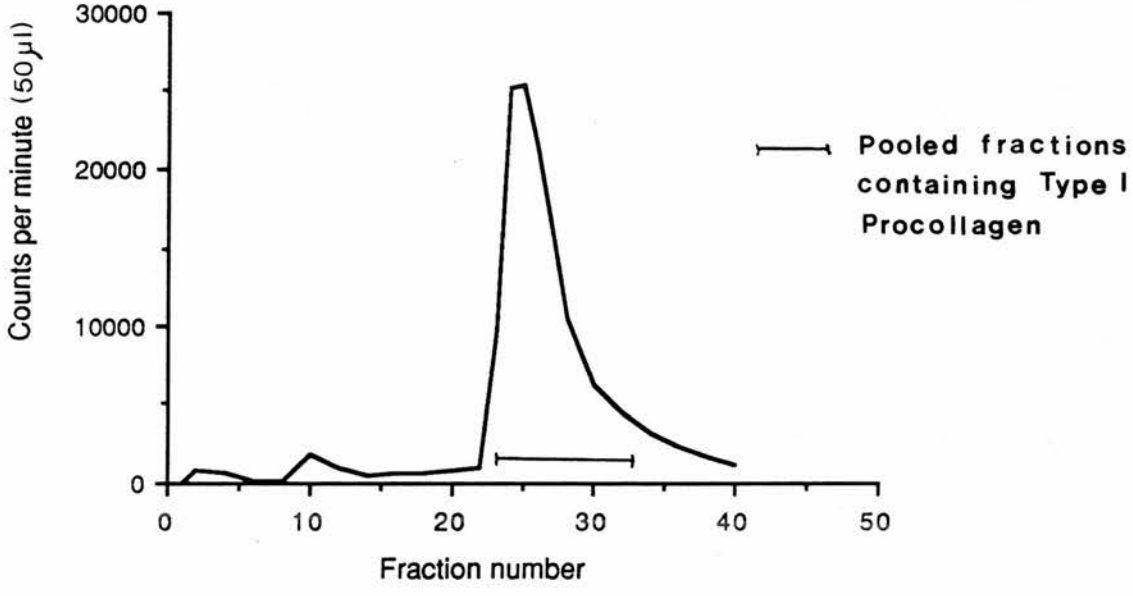

Figure 6.1 Elution profile of tendon type I procollagen on DEAE-Sephacel. Matrix-free tendon cells were labelled with 1 µCi/ml L-5-^{3}H proline for 5 hrs. The cells were removed and the proteins were precipitated with PEG-4000. After washing to remove the PEG and dialysis against 50 mM Tris-HCl pH 7.4/2 M urea/2.5 mM EDTA/10 mM NaCl, the radioactive proteins were applied to a column of DEAE-Sephacel and eluted with a gradient of 10-310 mM NaCl at a flow rate of 20 ml/hr. The fraction size was 8 ml.

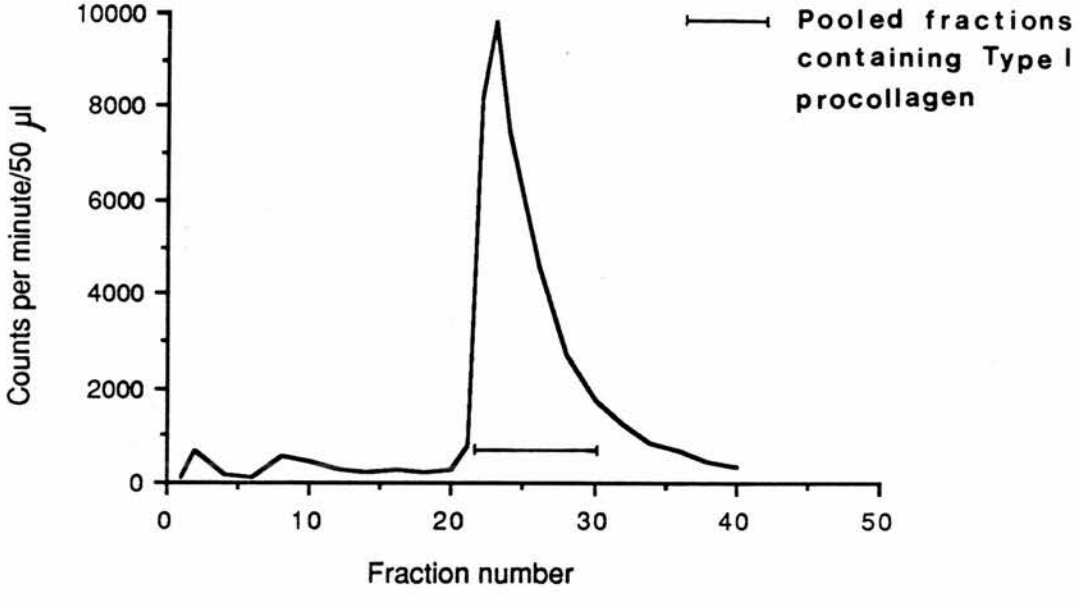

Figure 6.2 Elution profile of corneal type I procollagen on DEAE-Sephacel. Matrix-free corneal cells were incubated with 1 μCi/ml L-5-^{3}H proline for 5 hours, and the medium was then treated as described in Section 2.2. Note that the position of elution of the corneal procollagen is similar to that of the tendon procollagen.

containing 250 μg/ml ascorbate for 5 hours and the secreted proteins were precipitated using PEG-4000. Purification was achieved by ion-exchange chromatography on DEAE-Sephacel. Profiles showing the elution of tendon type I and corneal type I procollagen are shown in Figures 6.1 and 6.2 respectively.

It can be seen from the figures that the position of elution of the two type I procollagens are very similar, indicating that the two molecules have similar charge properties.

6.3.2 Characterization of Corneal and Tendon type I Procollagen

The yield of corneal type I and tendon type I procollagen was determined by measuring the levels of hydroxyproline in the samples (Section 2.3.1; Woessner, 1961; Berg, 1982). The standard deviation of the points on the standard curve was nearly always less than 2%, and so the amounts of hypro in the samples could be confidently calculated. By a simple calculation (given in Section 2.3.1) the concentration of tendon type I procollagen was determined to be 730 μg/ml and the concentration of corneal type I procollagen was 312 μg/ml. The specific activity of the tendon type I procollagen was 10,500 cpm/μg and that of the corneal procollagen was 8,500 cpm/μg. The total yields of tendon and corneal type I procollagens were 1.5 mg and 0.5 mg respectively.

The reason for the decreased yield of corneal type I procollagen is not clear.

The two procollagens were also characterized by SDS-PAGE (section 2.3.2). Type I procollagens from tendon and cornea were electrophoresed on 6% acrylamide gels under reducing conditions (Figure 6.3). It can be seen that the majority of the protein migrated as 2 bands identified as proα1(I) and proα2(I) by comparison with known intermediates (Butkowski, Noelken and Hudson, 1982;

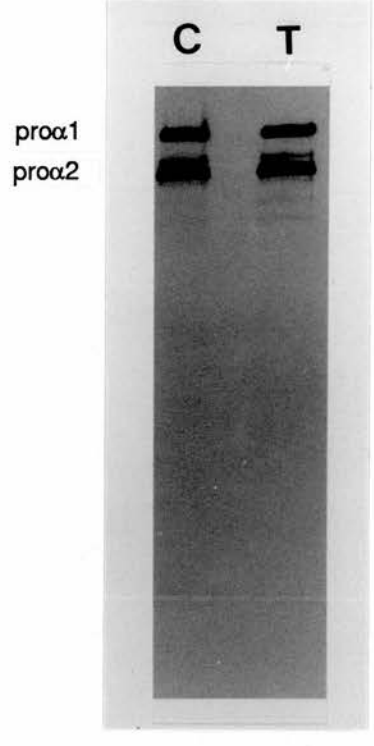

Figure 6.3 SDS-Polyacrylamide Gel Electrophoresis of tendon and corneal type I procollagens. The percentage of acrylamide was 6%. The gel was run at a constant current of 45 mA until the dye front was about 1 cm from the bottom of the gel (Section 2.3.2). The gel was then processed for fluorography as described (Section 2.3.3). C, corneal procollagen. T, tendon procollagen.

Prockop and Tuderman, 1982). A minor component migrated between the proα1(I) and proα2(I) chains, and this was identified as pCα1(I) (Figure 3.8; Butkowski, Noelken and Hudson, 1982; Prockop and Tuderman, 1982). This minor contaminant was present in all procollagen preparations, and by densitometry the pCα1(I) accounted for 7.5% of the collagenous proteins in both tendon and corneal procollagen.

The tendon type I and corneal type I proα1(I) and proα2(I) chains migrated at the same positions on SDS gels. This is in contrast to the results of previous workers. On 6% gels, acid soluble bovine tendon collagen migrated faster than bovine corneal collagen (Valli et al, 1986). Also, on 6% gels, chick tendon collagen migrated faster than chick corneal collagen (Kao and Foreman, 1980), whereas unhydroxylated procollagens showed no difference in mobility (Kao, 1985). Pepsin-extracted corneal type I collagen migrated slightly more slowly than skin type I collagen (Freeman, 1982b). Birk and Lande (1981) did not observe differences in the mobility of scleral and corneal type I collagens on SDS-gels of an unspecified acrylamide concentration. As unhydroxylated tendon and corneal procollagens migrated at the same position on 6% gels (Kao, 1985), it was suggested that the differences in mobility were due to the different levels of post-translational glycosylation.

However, no differences in the mobilities of tendon and corneal procollagen were ever seen in any of the preparations used in the present study.

6.3.3 Purification of Procollagen N-Proteinase and Procollagen C-Proteinase

Samples of the purified enzymes were provided by Dr D J S Hulmes. The C-proteinase was purified by the method of Hojima, van der Rest and Prockop (1985), except that the final purification on the Sephacryl S-200 column was

omitted. Procollagen N-proteinase was purified by modifications of the method of Tuderman, Kivirikko and Prockop (1978) and Tuderman and Prockop (1982) using whole tendons (Hojima, personal communication).

The N-proteinase was assayed by Dr Hulmes using the method given in Section 2.4.3 and the activity of the enzyme was calculated to be 700 U/ml. Using a similar assay (Section 2.4.2) the activity of the C-proteinase was calculated to be 280 U/ml.

6.3.4 Activity of the purified enzymes against tendon and corneal type I procollagen

Before producing collagen fibrils in vitro by using the purified N- and C-proteinases to cleave the propeptides from the procollagen molecule, it was necessary to compare the activities of the N- and C-proteinases on the corneal and tendon procollagen substrates.

Each of the procollagens was incubated with each of the enzymes at 37°C as described in Sections 2.4.2 and 2.4.3. Samples were removed at various times and assayed for activity by SDS-PAGE (Section 2.3.2). The activity of the enzymes was tested at a substrate concentration of 12 μg/ml. Tendon procollagen and corneal procollagen were each incubated separately with N-proteinase and C-proteinase each at 20 U/ml. The total assay volume was 200 μl. Care was taken to ensure that the final buffer composition was that of assay buffer (Sections 2.4.2 and 2.4.3), since variations in salt concentration are known to affect enzyme activity (Hojima, van der Rest and Prockop, 1985).

The samples were incubated at 35°C for 24 hrs. Control samples (corneal or tendon procollagen without any enzyme present) were also prepared and incubated for 24 hrs. At various time intervals 25 μl aliquots were removed. These aliquots were treated with 25 μl 'stop' solution as described in Sections 2.4.2 and 2.4.3. The aliquots were

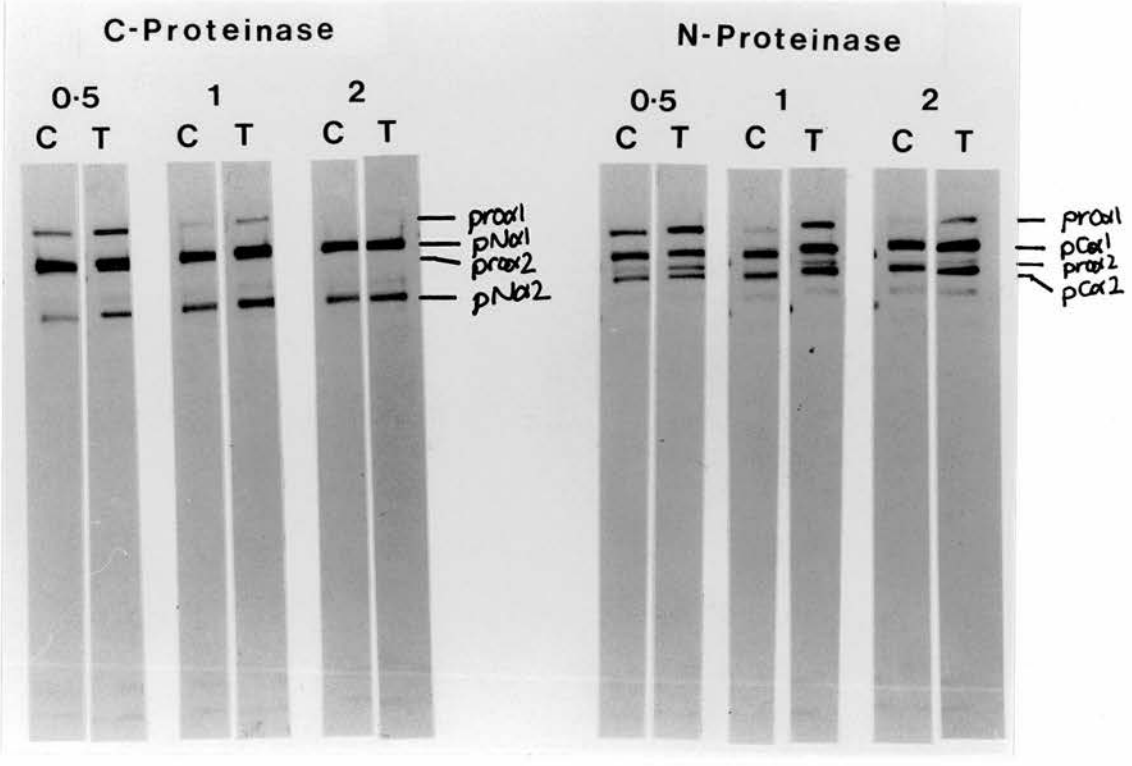

Figure 6.4 Time course of cleavage of tendon and corneal type I procollagen with procollagen N-proteinase and procollagen C-proteinase. The extent of cleavage of the procollagens by the enzymes is shown at 0.5, 1 and 2 hours. C, corneal procollagen. T, tendon procollagen. N-proteinase: samples cleaved with procollagen N-proteinase. C-proteinase: samples cleaved with procollagen C-proteinase.

analyzed by SDS-PAGE, fluorography and densitometry (Section 2.3.2, 2.3.3 and 2.3.5).

Quantitation of the fluorograms was difficult due to the presence of contaminating enzyme activities and a small amount of pCα1(I) chains. However, it can be seen from examination of the fluorograms that the cleavage of the corneal and tendon procollagens by the enzymes proceeded at the same rate (Figure 6.4). In other experiments where rapid assays were carried out as in Sections 2.4.2 and 2.4.3, the extent of cleavage by N-proteinase and C-proteinase was the same after 4 hours for both procollagens (not shown).

Hence, it was concluded that the corneal type I and tendon type I procollagen were cleaved by N-proteinase and C-proteinase at the same rate, within the limits of experimental error.

6.3.5 *In Vitro* Fibril Formation

Tendon and corneal type I procollagens and the N- and C-proteinases were dialyzed against fibril formation buffer (Section 2.4.4) at 4°C overnight, with one change. A rapid assay was carried out to determine the amount of enzyme activity remaining after dialysis (Sections 2.4.2 and 2.4.3). Assaying the N-proteinase activity which had not been dialyzed revealed that the activity was now 90 U/ml. This contrasts with the initial activity of 700 U/ml. The N-proteinase is known to be unstable (Hulmes, personal communication), so it seems that the activity had declined sharply during storage. After dialysis, the activity was 34 U/ml.

The undialyzed C-proteinase preparation had an activity of 260 U/ml, which is the same as the original preparation, indicating that the C-proteinase was more stable during storage. The activity had decreased to 38.5 U/ml after dialysis.

The dialyzed enzymes and substrates were incubated

together using a procollagen concentration of 120 μg/ml and enzyme activities were added to a final concentration of 10 U/ml for both N- and C-proteinase. The total volume of each sample was 100 μl, and the volume was made up with fibril formation buffer. The experiment was carried out in triplicate. In addition, duplicate samples of volume 50 μl containing the same proportions of substrate and enzymes were prepared for analysis by SDS-PAGE. The samples were mixed on ice and then incubated at 37°C for 24 hours. Control samples without enzymes were also incubated for 24 hours at 37°C. After 24 hours the samples for EM and SDS-PAGE were treated as described in Sections 2.4.4 and 2.4.5.

After centrifugation of the EM samples, it could be seen that there were differences in fibril formation for tendon and corneal type I procollagen. The pellets formed on centrifugation by the tendon samples were larger than the pellets formed by the corneal samples, which were scarcely visible.

A difference in the fibrillogenesis of tendon and corneal type I procollagens was confirmed by SDS-PAGE (Figures 6.5 and 6.6 respectively). The control samples (incubated without enzymes) were not processed to pN-, pC-collagen or collagen. The supernatants and pellets of the samples which were incubated with the enzyme were examined separately by SDS-PAGE, fluorography and densitometry. For tendon procollagen, it can be seen that virtually all of the sample was present in the pellet, and the procollagen was completely processed to collagen. The amount of collagen precipitated was 93-96% as determined by densitometry. However, the situation was completely different for the corneal procollagen. Complete processing also occurred, but most of the collagen remained in the supernatant (93.4-96.1%) and was not precipitated in the form of fibrils (Figure 6.6). Thus, corneal collagen seems to be more soluble than tendon type I collagen. Confirmation of this phenomenon was shown by liquid

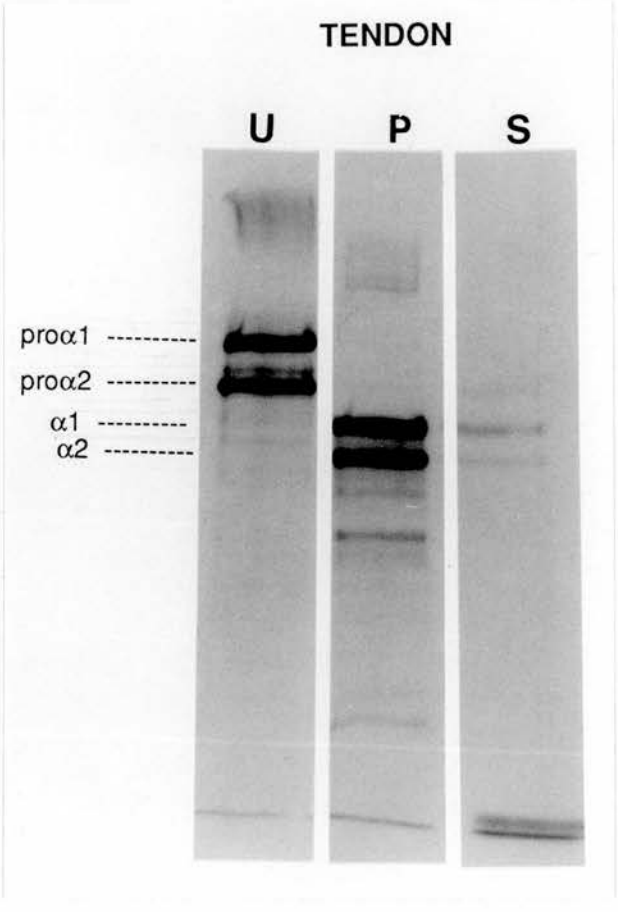

Figure 6.5 Collagen fibril formation in vitro of tendon type I procollagen. The procollagen was incubated with the N- and C-proteinases for 24 hrs at 37°C. The samples were then treated as described in Section 2.4.2 and analyzed by SDS-PAGE on 6% acrylamide gels. U, control type I procollagen incubated at 37°C without enzymes; P, sample which formed a pellet after centrifugation for 5 minutes at 11,600 g; S, sample which remained in the supernatant at 11,600 g for 5 minutes (Section 2.4.4).

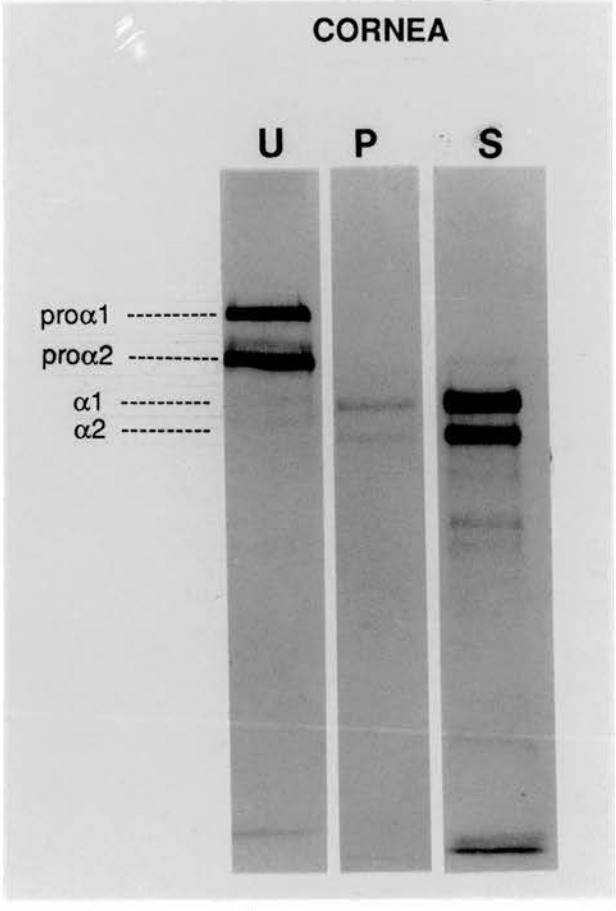

Figure 6.6 Collagen fibril formation in vitro of corneal type I procollagen. The procollagen was incubated with the N- and C-proteinases for 24 hrs at 37°C. The samples were then treated as described in Section 2.4.2 and analyzed by SDS-PAGE on 6% acrylamide gels. U, control type I procollagen incubated at 37°C without enzymes; P, sample which formed a pellet after centrifugation for 5 minutes at 11,600 g; S, sample which remained in the supernatant at 11,600 g for 5 minutes (Section 2.4.4).

scintillation counting of 50 μl aliquots of the supernatants of the samples taken for electron microscopy.

The samples of collagen from cornea and tendon which were precipitated were examined by electron microscopy. Electron micrographs of the fibrils formed from tendon collagen are shown in Figure 6.7. It can be seen that the material precipitated in the pellet consisted of D-periodic fibrils. The material precipitated by centrifugation of the processed corneal collagen, however, showed differences from the tendon samples (Figure 6.8). Very few fibrils were seen. Mostly small stained aggregates were observed, possibly corresponding to small aggregates of a few collagen molecules.

6.3.6 Lectin blotting

Type I collagens from tendon and cornea were shown to have similar physical and charge properties (Birk and Silver, 1983) and differed only in molecular weights, presumably due to differences in the amounts of post-translational glycosylation (Grant et al, 1969; Schofield, Freeman and Jackson, 1971). As levels of hydroxylysylglycosides have been implicated in the regulation of fibril diameter, it was important to determine that the procollagens synthesized in cell culture from tendon and corneal fibroblasts were glycosylated, and to determine the amounts of such glycosides.

Previous techniques for determining sugars are gas-liquid chromatography (glc) (Grant et al, 1969; Schofield, Freeman and Jackson, 1971); paper electrophoresis of alkaline hydrolyzates and glc (eg Schofield, Freeman and Jackson, 1971) and amino acid analysis (Schofield, Freeman and Jackson, 1971; Panjwani and Harding, 1978). In the present study it was not possible to determine sugars by separation on an amino acid analyzer. Thus it was decided to try to detect the presence of sugar by lectin blotting.

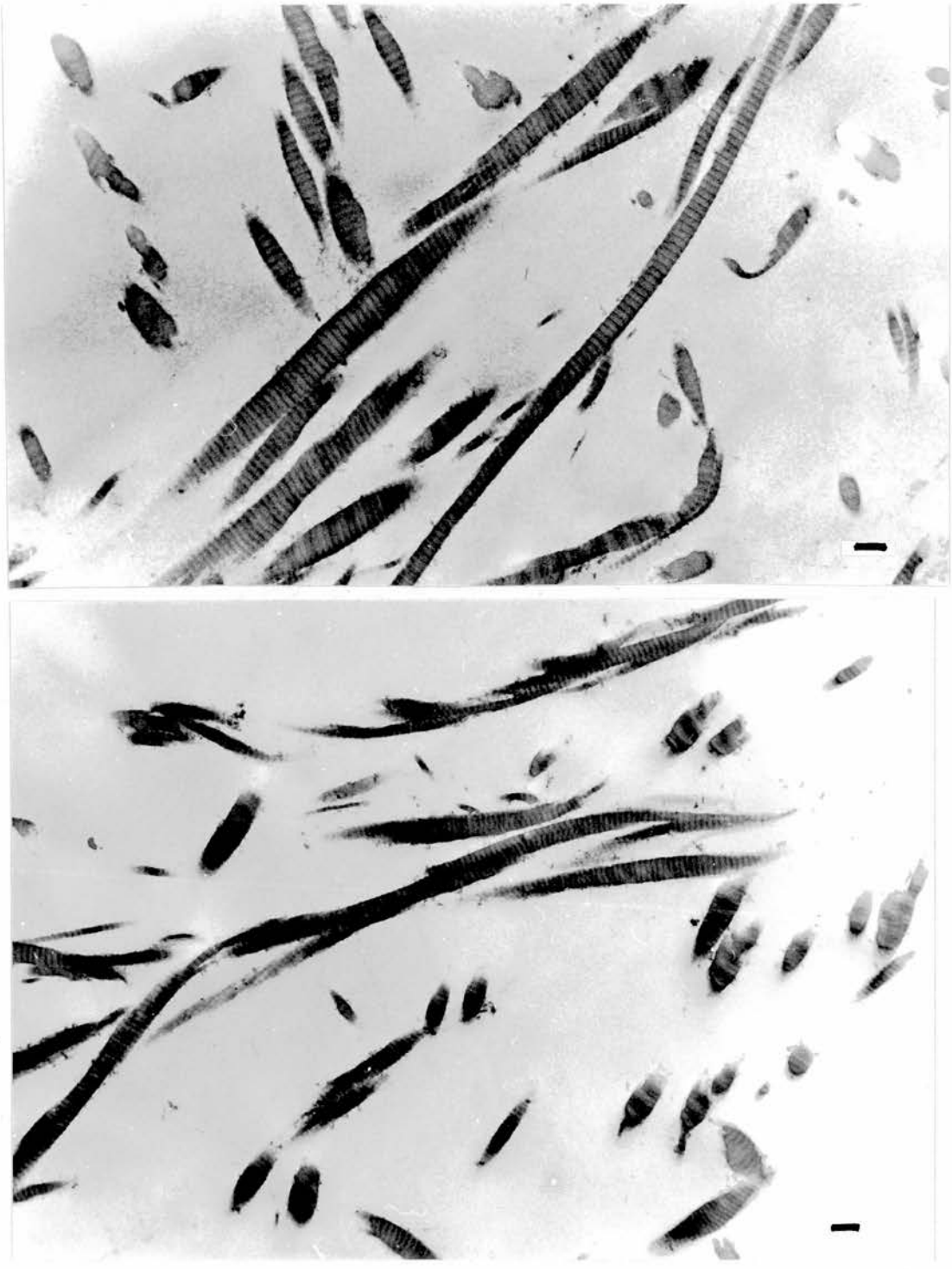

Figure 6.7 Electron micrographs of structures observed in the pellet formed after incubation of the tendon procollagen with the N- and C-proteinases (Sections 2.4.4 and 2.4.5). Magnification, x 20,000. Bar, 200 nm.

(Courtesy of Mrs C Cummings)
Top = 88E/1605
Bottom = 88E/1610

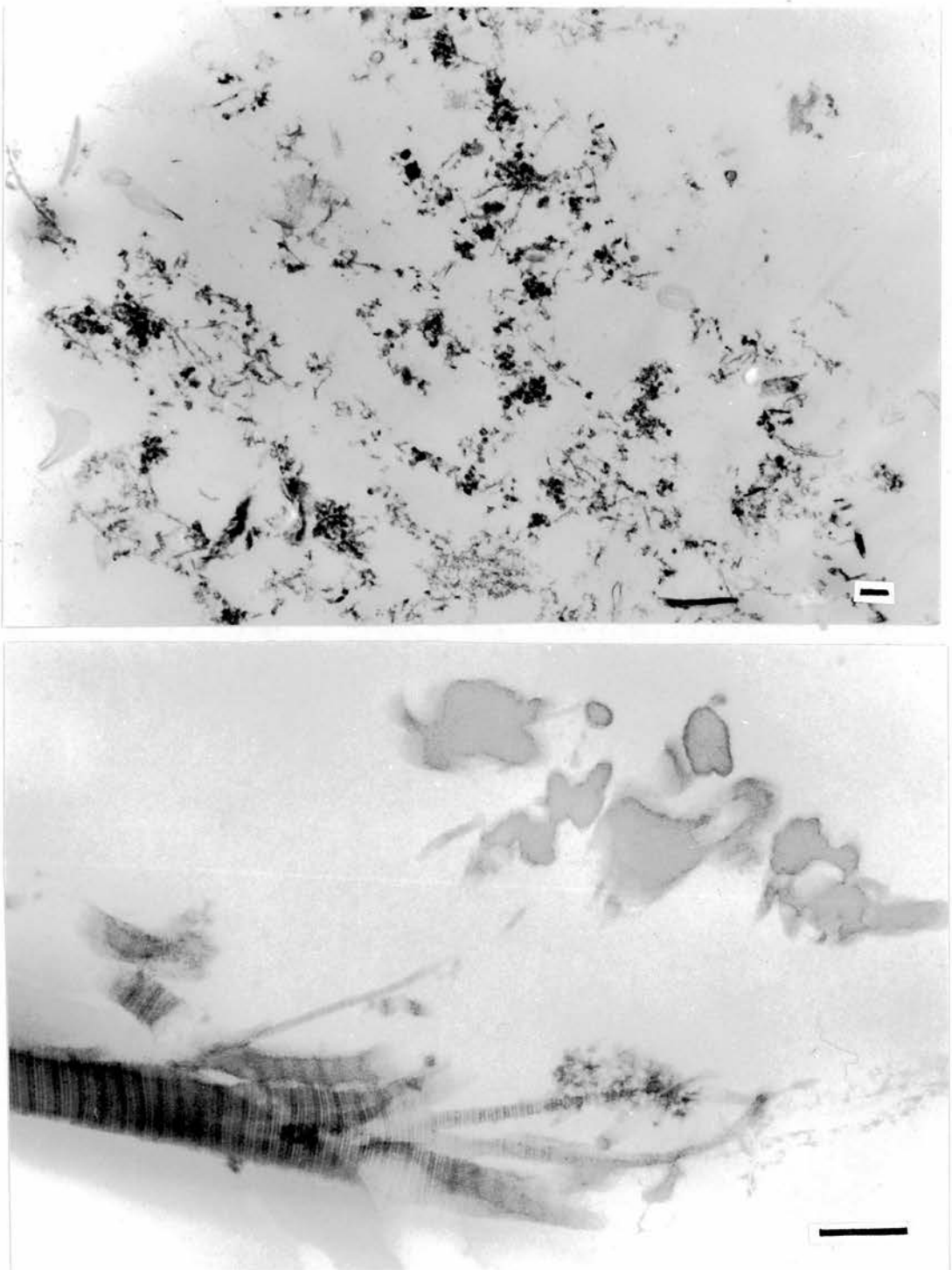

Figure 6.8 Electron micrographs of structures observed in the pellet formed after incubation of the corneal procollagen with the N- and C-proteinases (Sections 2.4.4 and 2.4.5). . Bar, 200 nm.

(Courtesy of Mrs C Cummings)
Top = 88E/1616 (x 20,000)
Bottom = 88E/1617 (x 61,500)

The blotting procedure was as described in Section 2.5.2.

Lectin blotting has previously been used to detect sugars on chromogranins A and B (Apps, Phillips and Purves, 1985). The streptavidin-biotin labelling system is based on the high affinity of egg white avidin ($k_D = 10^{-15}$ M) for biotin. The interaction is non-covalent and essentially irreversible. Use of streptavidin provides a means of detecting biotinylated probes and of amplifying reactions due to the high affinity of biotin for streptavidin.

Samples of pepsinized corneal and tendon procollagens and rat tail tendon collagen were electrophoresed on 6% acrylamide gels under reducing conditions (Section 2.3.2). Proteins were transferred to nitrocellulose and probed with viscumin (Section 2.5.2).

The lectin blot was exposed to X-ray film for 4 hrs at -70°C (Figure 6.9). Track 1 of Figure 6.9 corresponds to the biotinylated standard, which clearly has been detected by the ^{125}I-streptavidin. This indicates that the detection system was working normally. The non-biotinylated standards (tracks 2 and 6) were not detected by the viscumin; these samples were included as a negative control. Tracks 7, 8, 9 have been detected by the ^{125}I-streptavidin. These tracks contained rat tail tendon collagen (20 μg, 50 μg, 100 μg respectively). The ^{125}I-streptavidin detected biotinylated lectin bound to the α1 chain. No ^{125}I-streptavidin binding was found associated with the α2 chain. It is not clear why the lectin did not bind to the α2 chain.

Bands of radioactivity were detected in tracks 3 (pepsinized tendon procollagen, 20 μg), 4 (pepsinized tendon procollagen, 30 μg) and 5 (pepsinized corneal procollagen, 20 μg). At first sight it seemed that the lectin bound to the protein had been detected by ^{125}I-streptavidin. The pepsinized procollagen samples were ^{3}H-labelled, however, so it was possible that the X-ray film may also have detected ^{3}H decay. After fluorography of the

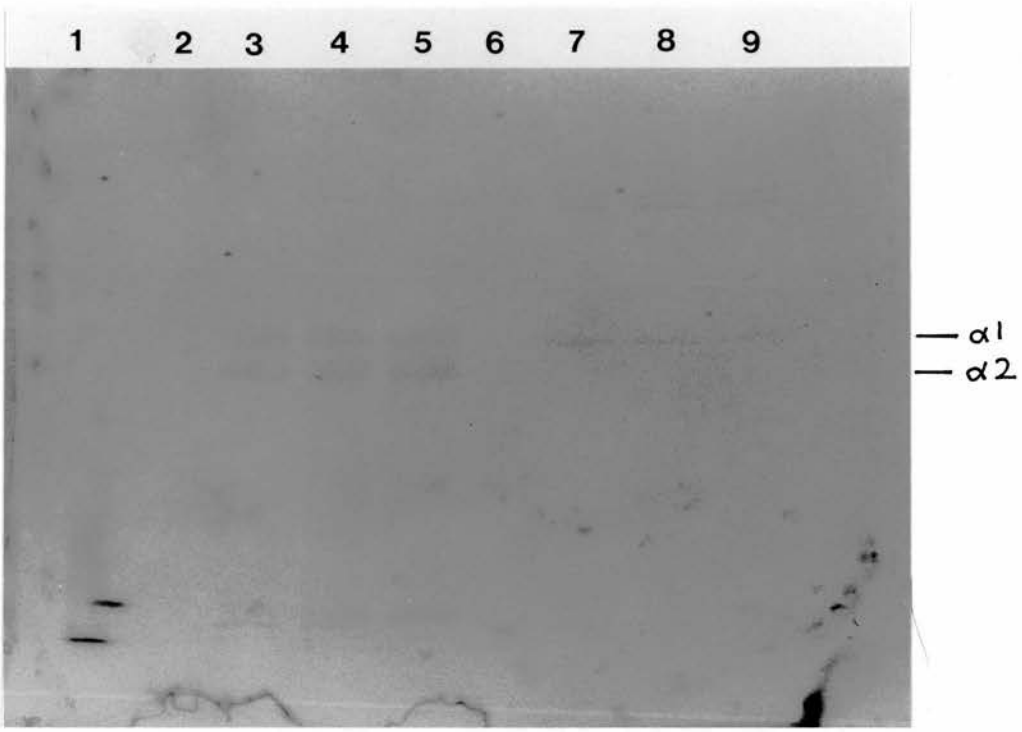

Figure 6.9 Lectin blot of pepsinized corneal and tendon procollagen samples which were electrophoresed on 6% SDS-PAGE, transblotted to nitrocellulose and probed with biotinylated viscumin (0.02 mg/ml). The biotinylated viscumin was detected with ^{125}I-labelled streptavidin (Section 2.5.2).
Track 1, biotinylated standard; tracks 2 and 6, non-biotinylated standard; track 3, pepsinized tendon procollagen (20 μg); track 4, pepsinized tendon procollagen (30 μg); track 5, pepsinized corneal procollagen (20 μg); track 7, rat tail tendon collagen (20 μg); track 8, rat tail tendon collagen (50 μg); track 9, rat tail tendon collagen (100 μg). The rat tail tendon collagen was a gift from Dr D R Shackleton.

gels used for transblotting, it was seen from the intensity of the ^{3}H-labelled bands that the efficiency of transfer from the SDS gel to the nitrocellulose was very low (Figure 6.10). To ensure that only ^{125}I decay was being detected, squares were cut out of the nitrocellulose blot for counting in an LKB gamma counter. The number of counts for the rat tail collagen samples was significantly greater than the background counts, confirming that the radioactivity was due to ^{125}I-streptavidin specifically binding to the rat tail tendon collagen samples. However, it was found that the number of counts per minute for the procollagen samples was about the same as the background sample (Table 6.1). Hence it was not possible to conclude that the pepsinized procollagen samples were glycosylated. Also, no differences in cpm between the two samples of rat tail tendon collagen were detected by gamma counting, even though the amounts loaded on SDS-PAGE differed by a factor of 2.5. Thus, this technique may not be capable of detecting differences in levels of sugar of different samples.

6.4 Discussion

Results of rapid assays showed that the purified N- and C-proteinases cleaved tendon and corneal procollagen at the same rate. Thus, it was decided to use an *in vitro* system to examine fibril formation from corneal and tendon procollagens by removal of the propeptides with the N-and C-proteinases. This system had not previously been used to to examine collagen fibril formation from corneal type I procollagen (Miyahara *et al*, 1982, 1984; Kadler, Hojima and Prockop, 1987).

Since the N- and C-proteinases cleaved the propeptides from the two procollagens at the same rate, this means that when tendon and corneal procollagens are incubated with the two enzymes, collagen will also be generated at the same rate. Thus, in this *in vitro* system any

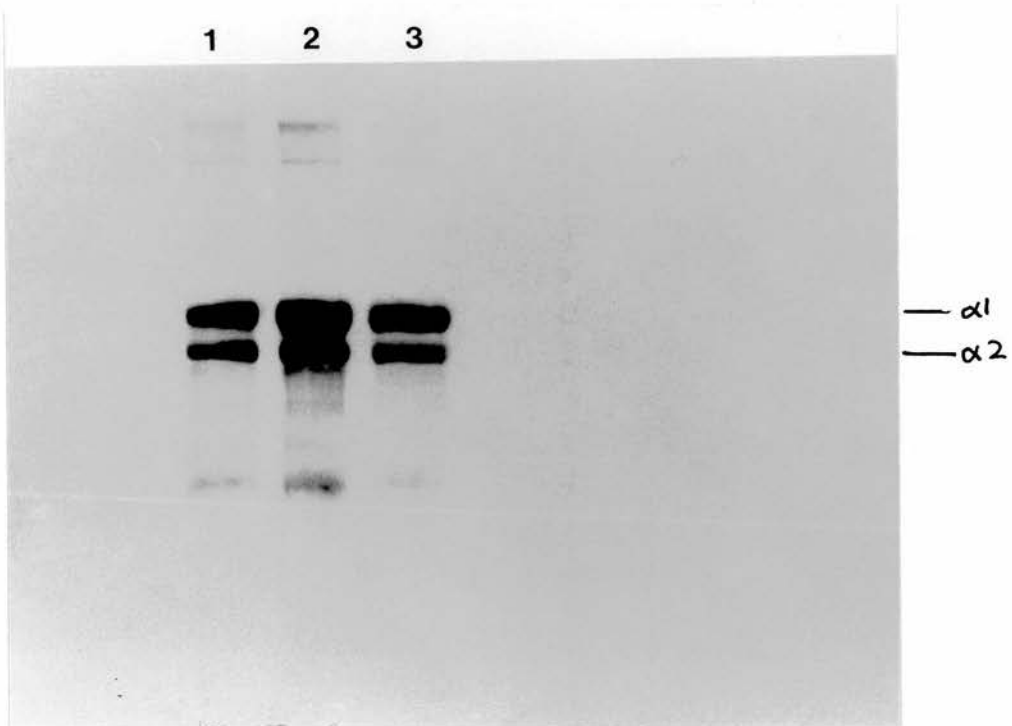

Figure 6.10 Fluorogram of the gel used for lectin blotting, after transblotting to nitrocellulose. The gel was then processed for fluorography (Section 2.3.3). Track 1, pepsinized tendon procollagen (20 μg); track 2, pepsinized tendon procollagen (30 μg); track 3, pepsinized corneal procollagen (20 μg).

Sample	Cpm	Error
Tendon Procollagen-20μg	16429	0.8%
Tendon Procollagen-30μg	14138	0.8%
Corneal Procollagen-20μg	16741	0.8%
Rat Tail Tendon Collagen-20μg	17585	0.8%
Rat Tail Tendon Collagen-50μg	17282	0.8%
Background	16754	0.8%

Table 6.1 Table of Gamma Counting.
Samples were transblotted onto nitrocellulose, probed with biotinylated viscumin and detected with ^{125}I-labelled streptavidin. Squares were cut out of the nitrocellulose and counted to detect ^{125}I binding in a gamma counter (LKB)

differences in the fibril forming process cannot be due to different initial concentrations of collagen.

Tendon and corneal procollagens (120 μg/ml) were incubated in a "physiological" buffer with 10 U/ml each of N- and C-proteinase at 37°C for 24 hours. After 24 hours, complete processing of the procollagens to collagen had occurred, as shown by SDS-PAGE. About 95% of the tendon sample was aggregated in the form of fibrils, whereas less than 5% of the corneal sample had aggregated. On examination by electron microscopy, it was seen that the tendon sample had formed a large pellet, which consisted of D-periodic fibrils. In contrast the corneal sample formed only a very small pellet and fibrils were present only occasionally. Stained aggregates were seen, but defined structures could not be discerned.

The fact that only a small percentage of the corneal collagen was precipitated in the form of fibrils indicated that the corneal collagen was much more soluble than the tendon collagen.

Corneal collagen formed fibrils 6-7 times more slowly than scleral type I collagen (Birk and Lande, 1981). The rate of the growth phase was not affected; the difference was in the length of the lag phase. However, the rates of both phases were later found to be slower (Birk and Silver, 1984b). The activation energies for both the lag and growth phases were larger for corneal collagen than for scleral collagen. Corneal type I collagen was found to form fibrils with a longer lag phase and a slower rate of growth than skin and tendon type I collagens (Valli et al, 1986).

The results obtained in the present study seem to extend the conclusions from the work quoted above. It is suggested that the higher activation energy of corneal fibril formation process is due to the increased solubility of the collagen observed here. The increased activation energy would result in a longer lag phase and possibly a slower rate of fibril formation. Thus, the EM

results could be explained by the increased solubility of the corneal collagen. The lag phase and growth phase might be prolonged, so that under conditions in which type I tendon collagen is known to form fibrils, the type I corneal collagen fibril forming process might be incomplete. In the EM, aggregates of stain were seen, but definite structures could not be discerned at the magnification used. Thus, it is suggested that these small aggregates may consist of intermediate forms of collagen fibril assembly: perhaps limiting initial aggregates, initial aggregates, and early linear fibrillar aggregates (Trelstad, Hayashi and Gross, 1976; Trelstad, Birk and Silver, 1982). If this is correct, it is suggested that incubation of the corneal samples for a longer period than 24 hours might have resulted in more fibrils being formed (Chapter 7).

Because the number of fibrils formed from corneal collagen was so small, it was not possible to determine a fibril diameter distribution. The tendon fibrils were approximately 200-300 nm in diameter. Hence, in the present study it was not possible to compare the diameters of fibrils formed by cleavage of type I tendon and corneal procollagen by cleavage with the N-and C-proteinases.

What factor, then, affects the solubility of the processed corneal collagen molecules? The factor most investigated in relation to corneal collagen fibrillogenesis is the higher levels of post-translational glycosylation of corneal type I collagen compared with scleral or tendon type I collagen (Grant et al, 1969; Schofield, Freeman and Jackson, 1971; Birk and Lande, 1981; Birk and Silver, 1984b; Valli et al, 1986; Amudeswari, Liang and Chakrabarti, 1987). It was pointed out by Birk and Silver (1984b) that the presence of mono- and di-saccharides attached to the hydroxylysyl side chain would result in a long hydrophilic side chain with a length of up to 0.8 nm. Thus, in order for fibrillogenesis to occur, these side chains must be redirected axially,

requiring a rearrangement of the inner hydration layer and possibly changes in hydrogen bonding with the molecular backbone of the collagen molecules. They also suggest that the increased glycosylation has increased the amount of hydrogen bonding with the inner water layer. It is suggested from the present study that these interactions increase the solubility of the corneal collagen and hence the activation energy for fibril formation (Birk and Silver, 1984b).

It has previously been suggested that the presence of glycosides in regions near the telopeptides affect conformational changes in the telopeptides proposed to be important in the initiation of fibrillogenesis (Helseth and Veis, 1981; Capaldi and Chapman, 1982). The presence of glycoside could prevent accretion because of the axial extension of the side chain. Glycosides have been identified in the CNBr fragment α1CB3, possibly in the overlap zone of α1CB6, and maybe in the hole zone of α1CB6 (Panjwani and Harding, 1978; Harding, Crabbe and Panjwani, 1980).

In order to test that glycosides were present, lectin blotting was carried out. Lectin blotting provides a means of detecting the presence of sugar on a collagen molecule, and the results are obtained quickly. The lectin used was viscumin, which detects galactose residues (Stirpe et al, 1982). The lectin detected glycosides on rat tail tendon collagen α1 chain, but not on the α2 chain, or on the pepsinized procollagen samples. It could not be concluded that the pepsinized procollagens were glycosylated as gamma counting did not reveal counts above background levels. This raises two possibilities:

a) either the procollagens were fully glycosylated and the lectin failed to detect the sugar due to poor efficiency of transfer of the pepsinized procollagen samples onto the nitrocellulose;

b) the procollagens produced in cell culture were not glycosylated, or the glycosylating enzymes were only

active at a low level in the cell culture system.

Hence, at the present time there is insufficient evidence to determine the glycosylation state of the procollagens and thus it is not possible to state definitely that increased solubility of the corneal procollagen is due to higher levels of post-translational glycosylation. As the molecules must have been normally hydroxylated in the cell culture system in order to be secreted, it could be assumed that the glycosylating enzymes must have been functioning as well. However, this assumption may not be reasonable, as the rates of hydroxylation and glycosylation are not similar (see Kivirikko and Myllyla, 1984, for references). It must be emphasized that the work presented in this chapter is preliminary and more work needs to be carried out in order to delineate the system (Chapter 7).

It was hoped that the lectin blotting technique would be able to detect relative amounts of protein. This seemed not to be the case as there was no change in intensity of the ^{125}I-exposed band over a 5-fold range of collagen. This needs to be further investigated.

If the increased solubility of the corneal collagen cannot be attributed to the presence of sugar, then what possible factors could be causing it? The presence of other collagen types can probably be eliminated, as there is no evidence on SDS-PAGE for any other collagen type except type I. The only other types that would be expected to be present in large amounts would be types V and VI. Types V and VI can probably be ruled out because of their different mobilities on gels from type I collagen.

Other candidates for affecting solubility are PGs and GAGs. It is unlikely that sulphated PGs copurified with the type I procollagen as they were shown to be separated from type I procollagen on DEAE-cellulose chromatography (Miller, 1971). The only other candidate for affecting solubility is HA. However, intact corneas did not synthesise any HA after stage 42 (16 days) (Toole and

Trelstad, 1971), so at 17 days, the stage used for preparation of the procollagens, no HA would be expected to be present. In contrast, in a detailed analysis, Hart (1978) found that at 20 days of embryonic development HA contributed 21% of the total ^{3}H GAG-associated radioactivity present, and more than this would be present at 17 days. Hence, if HA is not resolved from type I procollagen then the HA present could affect solubility. As GAG constitute 0.2-1 % of the total wet weight of the cornea, then HA would represent 0.04-0.2% of the total corneal wet weight. Clearly, more experiments need to be carried out to determine the nature of the component responsible for affecting the corneal type I collagen solubility.

CHAPTER 7

FINAL DISCUSSION

7.1 Discussion

In this work, possible factors involved in the regulation of collagen fibril diameter were investigated. Chapter 3 described the development of a pulse-chase protocol for determination of the rate constants of the steps of procollagen processing; proα to pCα (k_1), proα to pNα (k_2), pCα to collagen (k_3) and pNα to collagen (k_4). Chapters 4 and 5 described the application of the pulse-chase analysis to intact embryonic chick tendons and corneas respectively at different developmental stages and the results were discussed in relation to the fibril diameters. It was found that there was no obvious correlation between the route of processing and the fibril diameter. On SDS-PAGE, embryonic twelve day tendon type I procollagen appeared to be processed exclusively via a pC-collagen intermediate. In later stages of development, small amounts of pN-collagen were visible on SDS-PAGE as well as pC-collagen, and the amount of pN-collagen present increased as embryonic development progressed. The mean diameter of tendon collagen fibrils increased between 12 and 14 days of development, but did not change significantly after 14 days of development at the stages studied, although the spread of diameters increased. Hence these results are inconsistent with the data of Fleischmajer (1981, 1983, 1985, 1987a,b), which indicated that pN-collagen was associated with smaller fibrils, and pC-collagen was associated with wider fibrils. Weighted regression analysis of the pulse-chase data to determine the four rate constants of procollagen processing (Figure 3.1) showed that the data were consistent with a model of initial random cleavage at either end of the procollagen molecule, and rapid conversion of the pN-collagen intermediate to collagen. Rapid conversion of the pN-collagen intermediate to collagen results in very little pN-collagen being present on SDS-PAGE, and this explains the results of previous workers (Davidson, McEneany and

Bornstein, 1975; Morris et al, 1975; Uitto, Lichtenstein and Bauer, 1976; Leung et al, 1979). This is the first time that a detailed kinetic analysis of the conversion of procollagen to collagen has been carried out, and this work illustrates the necessity of carrying out a detailed kinetic study on this system. It is invalid and inadequate to deduce kinetic information solely from visual inspection of SDS-gels or fluorograms.

Due to the nature of the protocol described in Chapter 3, the data to be used for the regression analysis were somewhat scattered, and this occasionally led to difficulties in finding rate constants using the computer program. A wide scatter could result in final values of the rate constants determined by the minimization program that are not at the global minimum in the SSQs. In this case it is strongly recommended that a global search of the type described in Chapters 4 and 5 is carried out to ensure that the rate constants from the minimization program are the values obtained at the global minimum SSQ, and not at some local minimum.

The fact that the initial cleavage of procollagen in tendon is random, with similar rates of pC- and pN-collagen generation after the first cleavage, argues against a role for the intermediates in the control of fibril diameter. However, a more indirect role of the intermediates may be involved: for example, if pN-collagen is being cleaved rapidly and pC-collagen is being cleaved more slowly, then this might result in pC-collagen molecules being incorporated into collagen fibrils, possibly on the surface, with the C-propeptide inhibiting further addition of collagen molecules. Similarly, pN-collagen not cleaved by the N-proteinase might also be incorporated into the collagen fibrils in this way.

Since collagen fibrils in the cornea are of uniform and narrow diameter, it might be expected that the route of processing and the rate constants of processing would be different from those in tendon. By regression analysis of

the pulse-chase data, it was again found that initial cleavage at either end of the procollagen molecule was random. However, it was found that cleavage of pC-collagen to collagen was much faster than cleavage of pN-collagen to collagen at all the stages studied.

The results obtained in the present study argue against a direct role of the intermediates of processing in the control of fibril diameter. This is in contrast to the data obtained by Miyahara et al (1982, 1984), in which it was shown that cleavage of procollagen via a pN-intermediate resulted in narrow fibrils, whilst cleavage via a pC-collagen intermediate resulted in wide fibrils. This is possibly because these authors used different tissue sources of pN- and pC-collagen.

The possible role of O-glycosylation of collagen in the control of fibril diameter was investigated. Using an in vitro system in which tendon or corneal procollagen was incubated with purifed N- and C-proteinases, and the morphology of the collagen fibrils formed was investigated by electron microscopy. In such a system, tendon collagen formed D-periodic fibrils, whereas corneal collagen formed very few fibrils and mainly small aggregates with no discernible structure. These results were interpreted as being due to increased solubility of the corneal substrate, increasing the activation energy for fibril formation. It was not clear whether the increased solubility was due to the increased glycosylation of corneal collagen, as adduced by previous workers (Grant et al, 1969; Schofield, Freeman and Jackson, 1971), or whether copurification of a compound such as HA was responsible. Lectin blotting was employed in an attempt to determine quickly the relative amounts of glycosylation of the corneal and tendon type I procollagen, but this was unsuccessful.

In summary, then, a direct involvement of procollagen processing intermediates in the regulation of collagen fibril diameter was found to be unlikely in the present

study. It seems likely, although it could not be conclusively demonstrated, that the level of post-translational O-glycosylation is involved in the regulation of fibril diameter.

What other factors could be playing a role in the regulation of fibril diameter? PGs and GAGs have been proposed to play such a role (Section 1.6.4.1). Recent evidence, however, has indicated that in the cornea this may not be the case, as complete removal of all the GAGs had no effect on the fibril diameter (Bard, Bansal and Ross, 1988; Bansal, Ross and Bard, in press). Thus, it seems that factor most likely to affect fibril diameter in the cornea is the presence of large amounts of type V collagen. Type V collagen has an extra N-terminal globular domain that could sterically prevent the addition of any more collagen molecules. Only small amounts of type V collagen have been reported in tendon (Jimenez, Yankowsi and Bashey, 1978; Kao, Mai and Chou, 1982), so there may be insufficient type V collagen in tendon to control fibril diameter in this way. The results of Keene et al (1987) show that type III collagen is present on the surface of all tendon fibrils, both wide and narrow, although this has not been demonstrated for the embryonic chick. This argues against a role of type III collagen in the regulation of fibril diameter, as does the fact that type III collagen does not retain a bulky domain after processing which could enable it to limit the diameter of fibrils.

7.2 Ideas for Further Work

Preliminary results presented in Chapter 6 indicated that the fibril forming process was different for tendon and corneal collagen. The corneal type I collagen was more soluble than the tendon type I collagen and consequently it formed fibrils much more slowly than tendon type I collagen. Electron microscopic investigation revealed that

only small aggregates were formed, and very few fibrils. Thus, it is necessary to repeat the *in vitro* fibril formation experiments, incubating the corneal samples for a longer time to allow fibril formation to proceed, and indeed to see if fibrils can form under these *in vitro* conditions. Samples should be withdrawn from the incubation mixture at various times and examined by EM and spectrophotometry to investigate the fibril forming process. The critical concentrations for fibril formation for each of the two substrates could be determined (Kadler, Hojima and Prockop, 1987). The nature of the agent causing the increased solubility of the corneal type I collagen should be investigated further. The corneal and tendon type I procollagen should be further purified, perhaps by gel filtration (Mould and Hulmes, 1987) to exclude the possibility of HA or any other collagen types copurifying with the type I procollagens. The presence of sugars could be investigated further by lectin blotting, preferably with non-radioactive procollagens to prevent the ^{3}H from interfering with the ^{125}I. If non-radioactive substrates are used, then these could also be used to quantitate the amount of sugar present by amino acid analysis (Schofield, Freeman and Jackson, 1971). The preliminary results presented here indicated that lectin blotting could not be used to differentiate between relative amounts of sugar when different amounts of collagen were present. However, more work needs to be carried out to define the lectin blotting system, particularly as very little protein was transblotted onto the nitrocellulose.

A limitation of the present pulse-chase study was that the rates of individual reactions could not be determined, only the rate constants. Thus, the concentrations of the various components would need to be determined in order to find out the rates.

One explanation of the pulse-chase results is that corneal and tendon procollagen are cleaved by different enzymes, or by different forms of the same enzyme. It would be interesting to isolate N- and C-proteinases from cornea for comparison with the enzymes isolated from tendon. This could be achieved by purification to homogeneity, investigating the catalytic properties of the enzymes, and also their antigenic properties.

So far as the pulse-chase work is concerned, further work could be carried out with other tissues which contain mainly type I collagen to investigate the kinetics of type I procollagen processing in these tissues. Suitable tissues for such an analysis would be calvaria or long bones. However, this might be technically difficult due to the presence of mineral in the bones. Processing of type II procollagen could be investigated in a tissue such as sternum. Determining rate constants of type III procollagen processing would be difficult, as type III is found associated with type I collagen, and it would be difficult to resolve the components of type III procollagen processing from type I procollagen processing by SDS-PAGE.

The processing of type V procollagen is of interest, since types I and V are found in the same fibril in cornea. Type V procollagen could be purified and cleaved with the purifed type I-processing enzymes in order to see if these enzymes act against a type V substrate, and if so, how the kinetics of processing of type V procollagen compare with those of type I procollagen.

Note Added in Proof

A recent study (Fleischmajer *et al*, 1988) has investigated the correlation between fibril diameter in chick embryo tendons at various stages of development with the levels of type I pN- and pC-collagen, determined by Western blotting. It was found that the pN-:pC-collagen ratio increased from 0.82 at 14 days of development to 2.82 at 18 days of development, to 4.24 at 21 days and 5.45 at 9 days post-hatching. Meanwhile, the mean fibril diameter increased. Thus, the study of Fleischmajer *et al* (1988) reinforces the evidence presented in this thesis that there is no simple correlation between intermediates of procollagen processing and fibril diameter, because the amount of pN-collagen increases with days of development. If processing via a pN-collagen intermediate led to narrow fibrils, it would be expected that the pN-collagen:pC-collagen ratio would decrease with days of development.

However, the data of Fleischmajer *et al* (1988) are limited in their usefulness, as it is not possible to obtain quantitative data from Western blotting. Also, a detailed kinetic study of the sort presented in this work was not carried out.

It is possible to calculate a value for the steady-state ratio of pN-collagen:pC-collagen from the data obtained in Chapters 4 and 5:

$$k_2[\text{procollagen}] = k_4[\text{pN-collagen}]$$

$$k_1[\text{procollagen}] = k_3[\text{pC-collagen}]$$

Therefore, $$k_2 : k_1 = k_4[\text{pN}] : k_3[\text{pC}]$$

and $$[\text{pN}] : [\text{pC}] = k_2 . k_3 : k_1 . k_4$$

Values for the pN:pC ratios were calculated for the tendon and cornea data and are presented in Table 7.1. It was not possible to calculate values for the 12 day tendon data, the 19 day tendon α1-type chains, and the 14 day cornea α1-type chains as the standard deviations of the rate constants were too large to allow a value for the ratio to be calculated. It seems that the value of the

pN:pC ratio is greater in cornea than in tendon. The ratio in cornea appears to decrease between 14 and 17 days of development (as expected), while the ratio for the α2-type chains in tendon seems to increase, in accordance with the data of Fleischmajer et al (1988).

	CORNEA	TENDON
12 days		
α1	4.3-15	N.D.
α2	1.6-4.3	N.D.
14 days		
α1	N.D.	0.08-0.6
α2	2.2-6.1	0.1-0.5
17 days		
α1	2.5-8.3	0.1-0.2
α2	0.7-2.1	[a] 0.5-5.5 [b] 0.9-6.6
19 days		
α1	N.A.	N.D.
α2	N.A.	0.4-2.8

Table 7.1 pN/pC ratios obtained for each of the developmental stages in cornea and tendon. The figures represent the lowest and highest possible values of the ratio for the α1 and α2 chains at each stage, determined as stated in the text. N.A. not applicable. N.D., could not be determined. [a] = low k_2, low k_4 values for the 17 day α2-type chains; [b] = high k_4 values for the 17 day α2-type chains.

APPENDIX 1 - COMPOSITION OF MEDIA

1. Hanks' Balanced Salt Solution (HBSS)

Component	Amount (g/l)
$CaCl_2$ (anhydrous)	0.14
KCl	0.40
KH_2PO_4	0.06
$MgCl_2.6H_2O$	0.10
$MgSO_4.7H_2O$	0.10
NaCl	8.00
$NaHCO_3$	0.35
$NaH_2PO_4.7H_2O$	0.09
D-Glucose	1.00

2. Minimum Essential Medium (MEM)

Component	Amount (mg/l)
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4.7H_2O$	200.00
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4.H_2O$	140.00
D-Glucose	1000.00
Phenol Red	10.00
L-Arginine.HCl	126.00
L-Cystine	24.00
L-Glutamine	292.00
L-Histidine.HCl.H_2O	42.00
L-Isoleucine	52.00
L-Leucine	52.00
L-Lysine.HCl	72.50
L-Methionine	15.00
L-Phenylalanine	32.00
L-Threonine	48.00
L-Tryptophan	10.00
L-Tyrosine	36.00
L-Valine	46.00
D-Ca Pantothenate	1.00
Choline Chloride	1.00
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

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